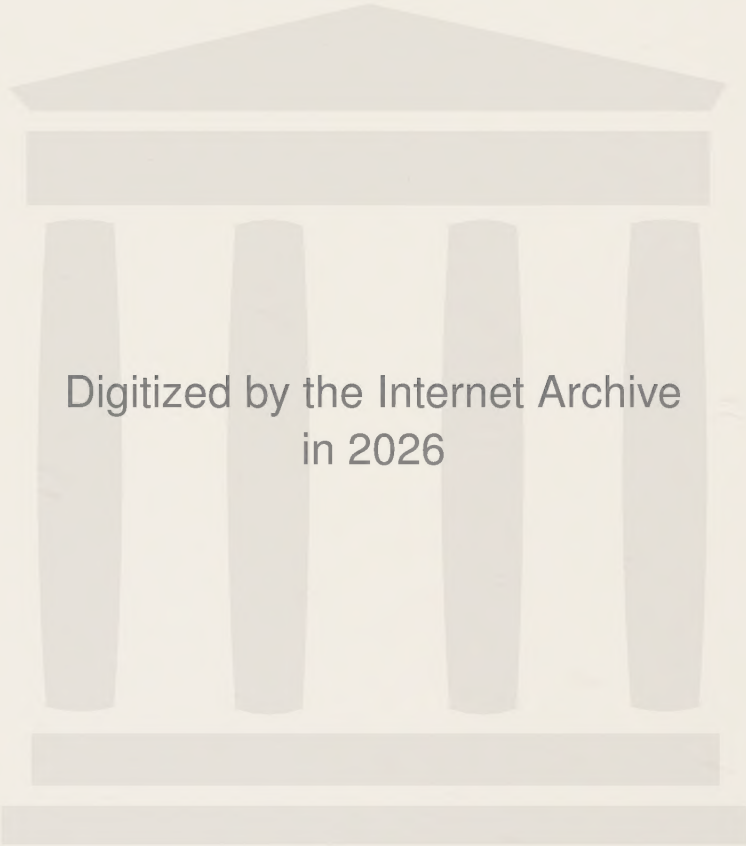


ON HAEMOPHILIA
WITH SPECIAL REFERENCE TO
GENETIC VARIATION PRENATAL DIAGNOSIS
AND DETECTION OF ANTICOAGULANTS

Rolf Ljung

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ON HAEMOPHILIA
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PRENATAL DIAGNOSIS AND DETECTION OF ANTICOAGULANTS

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Abstract Immunoradiometric assays (IRMAs) were developed for determining the two components of coagulation factor VIII, i.e. the von Willebrand factor (VIII:Ag) and the antihaemophilic factor A antigen (VIII:Cag). The assays were used, together with an IRMA for factor IX antigen (IX:Ag), for characterization of genetical variants of haemophilia (hf) A and B, for prenatal diagnosis and for detection of anticoagulants in hf A. The antihaemophilic factor A (VIII:C) was separated by antigen-antibody chromatography from VIII:Ag and it was demonstrated that the two components differed from each other in immunological reactivity, and thus confirmed the dual nature of the factor VIII complex. Of 15 patients with severe hf A all, but one lacked demonstrable VIII:Cag when examined with IRMAs utilizing two different antibodies. A genetically determined molecular variation was found when 54 patients with moderate or mild hf A were examined. Three subtypes were distinguished. According to an IRMA of IX:Ag, 17 of 18 patients with severe hf B lacked or had only tiny amounts of IX:Ag. In 32 patients with moderate and mild forms, IX:Ag was demonstrable either in amounts equal to IX:C or in larger or even normal amounts. Prenatal evaluation of 16 fetuses at risk for hf A and 3 for hf B by determination of VIII:Cag and IX:Ag respectively, in fetal blood, revealed that 5 were affected with hf A and 3 with hf B. Examination post abortem or post partum confirmed the diagnosis. The IRMA devised for detection of inhibitors in hf A was 20 times as sensitive as a conventional neutralization assay. Antibodies were detected with this method, but not with neutralization assay in 3 of 15 cases studied.		
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by

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Measurement of antihaemophilic factor A antigen (VIII:CAg) with a solid phase immunoradiometric method based on homologous non-haemophilic antibodies.
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- III Ljung R, Holmberg L, Nilsson I-M.
Inheritable molecular variants of moderate and mild haemophilia A.
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- IV Ljung R, Holmberg L.
F.VIII:CAg in haemophilia A - a comparison between IRMA:s using haemophilic and spontaneous antibodies.
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- V Ljung R, Holmberg L.
Genetic variants of haemophilia B detected by immunoradiometric assay - implications for prenatal diagnosis.
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INTRODUCTION

The most common and clinically important congenital bleeding disorders are haemophilia A, haemophilia B and von Willebrand's disease. Haemophilia A and B are due to a deficiency of coagulation factors VIII and IX, respectively (1-6). Both diseases have an identical clinical picture and the same recessive X-linked mode of inheritance, i.e. they affect males and are transmitted by females who are not themselves bleeders (5, 7-11).

Both haemophilia A and B are classified as severe, moderate or mild according to the activity of respectively factor VIII and factor IX in plasma (6, 12). The different forms are inherited as separate entities. On the basis of immunological properties of factors VIII and IX, a further heterogeneity was suggested early in both haemophilia A and B (13-15).

A difficult problem in haemophilia is the diagnosis of the carrier status in females (16-17). Genetic counselling in haemophilia has therefore been difficult, especially since the only safe preventive procedure for a woman has been either to refrain from having a child or to consent to interruption of any pregnancy with a male fetus.

The management of haemophilia has been greatly improved during the last two decades thanks to the development and clinical use of factor VIII and IX concentrates (18-20). These are used for substitution therapy of bleeding episodes and as continuous prophylactic treatment in childhood and adolescence (21). A dreaded complication of treatment is the development of antibodies, so-called anticoagulants, against factors VIII and IX, which make specific treatment difficult or even impossible (22-23).

This thesis deals with these 3 aspects of haemophilia: The genetically determined molecular variants of haemophilia A and B, genetic counselling and prenatal diagnosis of haemophilia, and, thirdly, with methods for early and safe detection of development of anticoagulants.

Biochemistry and nomenclature

It is now fairly well established that factor VIII is a molecular complex of two components (24-28, paper I). One of the components is the antihaemophilic factor A (AHF, factor VIII), the part which is defective or missing in haemophilia A. This factor is now usually designated VIII:C. It takes part in the first stage of the coagulation process, the activation of factor X. The other component is the von Willebrand factor (VIII:vWF, VIIIIR:Ag when determined immunologically), which is the major part of factor VIII. This component takes part in the primary haemostasis since it is necessary for adhesion of platelets to injured areas of a vessel wall. In addition, it serves as a carrier protein for VIII:C (29). VIII:vWF is the part which is defective or missing in von Willebrand's disease.

The synthesis of antihaemophilic factor A is controlled by an X-chromosomal gene. Little is known about its biochemical properties. It is thought to be a glycoprotein, but opinions differ about its M_r with estimates ranging from 25 000 to 285 000 (25, 27, 30-32). Its site of synthesis is not known. The plasma concentration is estimated to be 0.1-0.2 $\mu\text{g/ml}$ (29).

The synthesis of the von Willebrand factor is controlled by autosomal genes. There is evidence that it takes place in endothelial cells (33-35) and in megakaryocytes (36). According to recent studies, VIII:vWF is a glycoprotein with a basic subunit with M_r approx. 0.2×10^6 . It circulates

in plasma as series of polymers, the smallest probably being the tetramer (M_r 0.85×10^6), while the largest polymers have M_r around 12×10^6 (37, 38). The plasma concentration is estimated to be 5-10 $\mu\text{g/ml}$ (29).

Factor IX is synthesized in the liver by a vitamin K dependent process (39) and is controlled by an X-linked gene, such as VIII:C. It has been isolated from human plasma and is a single chain glycoprotein with a M_r of 57 000 (40, 41). The plasma concentration has been calculated to be 3-5 $\mu\text{g/ml}$ (41). Factor IX is activated by proteolytic cleavage to factor IX_a, which forms part of the factor X activating complex.

Definitions

- | | |
|------------|--|
| VIII:C | Factor VIII coagulant activity (antihaemophilic factor A) - the activity deficient or lacking in haemophilia A. |
| VIII:CAg | Factor VIII coagulant antigen - the antigen associated with that part of the factor VIII complex which possesses VIII:C |
| VIIIIR:Ag | Factor VIII related antigen - the antigen related to the von Willebrand factor part of the factor VIII complex |
| VIIIIR:RCF | Factor VIII related ristocetin cofactor activity - the activity of the von Willebrand factor part of the factor VIII complex, as measured <i>in vitro</i> with a test based on the aggregation of platelets by the antibiotic ristocetin |
| IX:C | Factor IX coagulant activity (antihaemophilic factor B) - the activity deficient or lacking in haemophilia B. |
| IX:Ag | Factor IX antigen |

THE PURPOSES OF THIS INVESTIGATION WERE:

- 1 To ascertain whether factor VIII really has a complex dual structure and, if so, to develop sensitive immunological methods for the separate determination of each of the two components of the complex, i.e. the factor VIII related antigen (VIII:Ag) and the factor VIII coagulant antigen (VIII:CAg).
- 2 To find out whether such methods and a similar method for IX:Ag can detect molecular variants of haemophilia A and B, and if so, whether these are genetically determined.
3. To assess, as a consequence of item 2, the value of immunoradiometric assays of factors VIII and IX in prenatal diagnosis of haemophilia A and B.
- 4 To develop a sensitive method based on an immunoradiometric assay, for detection of anticoagulants in haemophilia A.

METHODS

Collection of blood. Citrated plasma, obtained and prepared as described elsewhere (42), was immediately frozen and stored at -80°C until examined. Serum was obtained by letting whole blood stand for 2 h at room temperature.

Bleeding time (Paper III). This was measured according to the method of Ivy, as modified by the Simplate technique (43, 44).

Factor VIII coagulant (clotting) activity (VIII:C) was determined with a one-stage recalcification assay, which measures the ability of a patient's plasma to correct the defect in severe haemophilia compared with that of normal plasma (42, 45). Freeze-dried plasma (7th British Standard for factor VIII, 77/520) was used as the standard for the assay. The inter- and intraassay coefficient of variation is 10%. Normal range 60-160% (U/dl).

Purification of factor VIII (Paper I). This was purified by gel chromatography of cryoprecipitate, prepared from 400 ml normal plasma, on Sepharose 6 B according to van Mourik and Mochtar (46).

Preparation of antiserum against VIIIR:Ag (Paper I).

A factor VIII preparation (Hemofil, Hyland) was chromatographed on Sepharose 6 B according to van Mourik and Mochtar (46). Rabbits were injected with 1 ml of the void volume peak and a high antibody titre was achieved after four injections. After absorption with plasma from a patient with severe von Willebrand's disease type I, the antiserum gave one line with normal plasma, but none with von Willebrand's disease plasma in double diffusion. The antiserum had only a very slight inhibitory effect of VIII:C when mixed with normal plasma. IgG from the antiserum was prepared according to Steinbruch and Audran (47).

Factor VIII related antigen (von Willebrand factor, VIIIR:Ag) was determined in two ways with the use of the prepared rabbit antiserum:

1. Quantitative electroimmunoassay (Laurell) (48, 49). Normal range 40-175%.

2. Immunoradiometric assay (IRMA) essentially as described by Ruggeri et al (50), but as modified by Holmberg & Ljung (Page 14, paper I).

Ristocetin cofactor activity (VIII:RCF) (Paper I) was determined according to Zuzel et al (51). The assay measures the ability of factor VIII, in the presence of the anti-biotic Ristocetin, to aggregate platelets, which is believed to be a functional measure of the von Willebrand factor.

Factor VIII coagulant antigen (VIII:CAg) was determined with two immunoradiometric assays (IRMA) detailed in papers II and IV and on page 15.

Quantitative determination of inhibitors against VIII:C by neutralization assay (Paper VII) was performed according to Holmberg & Nilsson (52). In this method various dilutions of the plasma to be tested and a blank were incubated for 2 hours with a factor VIII concentrate. After incubation the residual VIII:C was determined. The inhibitor activity of plasma is expressed as the number of units of VIII:C inactivated by 1 ml of the plasma (one unit of VIII:C is defined as the amount present in 1 ml pooled normal plasma). One inhibitor unit in this method corresponds approximately to 2.5-3 units in the Bethesda method (53).

Factor IX coagulant (clotting) activity (IX:C) (Paper V) was determined with a one-stage recalcification method similar to that of VIII:C assay (42). Normal range 60-140%.

Factor IX coagulant antigen (IX:Ag) (Paper V) was determined with an immunoradiometric assay based on an antibody which arose in a haemophiliac (54). In short, IgG from the immune serum that had an inhibitor titre of 50 U/ml was isolated with Protein-A-Sepharose chromatography and labelled with ^{125}I . Specific labelled antibody to factor IX was purified by complexing the labelled IgG with a factor IX concentrate (Preconativ, KABI), which had been immobilized on CH-activated Sepharose 4 B. After incubation and washing procedures, the specific labelled antibody was eluted

with glycine-HCl buffer, pH 3. Assay technique. The assay was performed as a solid phase two-site technique, as described for VIIIIR:Ag (page 14). When normal plasma was tested the dose-response curve, plotted in a lin-log scale, was almost linear for dilutions 1/128-1/2048, with an amount of bound radioactivity ranging from 25%-3% of the amount added. Normal range 60-160 U/dl. Coefficient of correlation between IX:C and IX:Ag in normals 0.74 ($P < 0.001$). Sensitivity limit 0.025 U/dl. Inter- and intraassay coefficients of variation at low dilution of antigen (1:2000) was less than 10%.

SDS-polyacrylamide gel electrophoresis (Paper I) was performed according to Weber & Osborn (55).

P & P (Paper V) was measured according to the method of Owren & Aas (56). Normal range 80-120%.

Thrombotest (Paper V) was measured by Thrombotest reagent (Nyegaard, Oslo, Norway). Normal range 80-120%.

One-stage prothrombin time (Paper V) was determined with the method of Owren using thromboplastin prepared from human and bovine brains (57). Normal range 14-17 s.

CLINICAL MATERIAL

Paper II. The study group consisted of 13 patients with severe, 4 with moderate, and 4 with mild, haemophilia A and two patients with severe von Willebrand's disease. Citrated plasma and serum were also prepared from 5 normals.

Paper III. The study group consisted of 54 patients, who belonged to 28 kindreds with moderate or mild haemophilia A.

Paper IV. The material consisted of 47 patients, with haemophilia A. The disease was severe in 15, moderately severe in 11 and mild in 21. Most of the patients were also included in the clinical material described in papers II and III. Twenty-two normals were investigated to obtain reference values.

Paper V. The study group consisted of 50 patients with haemophilia B. They belonged to 29 kindreds. The disease was severe in 18, moderately severe in 9 and mild in 23.

Paper VI. In order make a prenatal diagnosis of haemophilia, fetal blood was sampled in the 18-20th gestational week from 16 male fetuses at risk for haemophilia A and from 3 fetuses at risk for haemophilia B. The fetal blood samples were obtained at foetoscopy by the foetoscopic teams in Copenhagen and Lund.

Paper VII. The study group consisted of 15 haemophilia A patients, most of them children, with known inhibitors of VIII:C, 10 multitransfused patients with severe haemophilia A without known inhibitors and 10 normals. Three non-haemophilic patients, aged 63, 69 and 72, who had spontaneously developed inhibitors against VIII:C were also investigated.

THE DUAL NATURE OF THE FACTOR VIII COMPLEX AND IMMUNO-
RADIOMETRIC ASSAYS OF VIIIIR:Ag and VIII:CAg - Papers I, II, IV

Background

In the beginning of the 70s it became possible by agarose gel chromatography to purify a protein possessing VIII:C activity (46, 58, 59) and to raise heterologous antisera against this protein fraction. Immunological analyses using such precipitating rabbit antisera as well as chromatographic studies (60) showed that patients with haemophilia A had this protein, which cross-reacted, but lacked VIII:C activity (24, 60, 61). The content of the protein in von Willebrand's disease was, however, decreased. When measured immunologically it was called factor VIII related antigen (VIIIIR:Ag). It can be quantified with an electroimmunoassay (24, 52), an immunoradiometric assay (50, 62), or a radioimmunoassay (63). It can also be measured in a functional test by its ability, in the presence of the antibiotic Ristocetin, to cause platelet aggregation, so-called factor VIII related ristocetin cofactor activity (VIIIIR:RCF) (64).

Antibodies which neutralize VIII:C activity can, though rarely, be obtained from multitransfused haemophiliacs and also from other patients. Such antibodies do not form immunoprecipitates (13), and do not interact with VIIIIR:Ag (65-66) or VIIIIR:RCF (64).

A concept evolved in the middle of the 70s was that the protein fraction with VIII:C activity, which could be purified from plasma, was a complex of 2 components: the antihaemophilic factor (VIII:C) and the von Willebrand factor (VIIIIR:Ag).

Some workers were able to dissociate VIII:C from VIIIIR:Ag and VIIIIR:RCF by gel chromatography at high ionic strength (25, 26, 67, 68). Others, however, questioned whether the dissociated VIII:C might not be a proteolytic cleavage product rather than a native protein (28, 69). When the present work was begun, this question was still debatable.

The purpose of this part of the work was to cleave VIII:C from VIIIIR:Ag and to ascertain with a sensitive immunoradiometric assay (IRMA) whether the dissociated VIII:C lacked the antigenic properties of VIIIIR:Ag. A second purpose was to develop a separate immunological method for determination of the antigen associated with VIII:C, factor VIII coagulant antigen (VIII:CAg).

Results

a) IRMA of VIIIIR:Ag (Paper I)

An IRMA of VIIIIR:Ag was developed as detailed in Paper I. Factor VIII was purified and an antiserum raised in rabbits (see methods, page 9). The antiserum was shown to be specific by absorption with plasma from a patient with severe von Willebrand's disease.

Whole IgG was labelled with ^{125}I . Specific, labelled antibodies were purified by a procedure that includes complex formation of the labelled IgG with factor VIII and isolation of the complex by Sepharose 6 B chromatography and dissociation of the complex by chromatography on Sephadex G-200.

The assay is performed as a solid phase two-site technique in three steps. In the first, polystyrene tubes are coated with unlabelled antibody and in the second the

plasma to be tested is added in various dilutions. In the third step purified ^{125}I labelled antibody is added. The tubes are washed after incubation and counted in a gamma counter.

The limit of detection of VIIIIR:Ag is 0.05 U/dl (approx. 0.5 $\mu\text{g/dl}$) (one unit being the amount of VIIIIR:Ag present in 1 ml of normal human pooled plasma). Normal range 40-175 U/dl, the interassay coefficient of variation at a concentration of 25 U/dl is 11%.

b) Separation of VIII:C from VIIIIR:Ag (Paper I)

A rabbit antibody to VIIIIR:Ag without VIII:C neutralizing activity was immobilized on a Sepharose 4 B matrix. Pure factor VIII was added to the column and VIIIIR:Ag as well as VIII:C was bound to the matrix. VIII:C was eluted by increasing the ionic strength of the buffer and was obtained free from immunoradiometrically detectable VIIIIR:Ag and free from VIIIIR:RCF. The preparation could be activated, although to a lesser degree than VIII:C in plasma, by trace amounts of thrombin. When stabilized with albumin it could be frozen and thawed with virtually undiminished activity. No protein was spectrophotometrically detectable in the VIII:C containing preparation, although VIII:C was 4 times that of normal plasma. When plasma was used as starting material, VIII:C, without VIIIIR:Ag, could still be obtained, but was then contaminated with other proteins.

c) IRMA:s of VIII:CAg (Papers II and IV)

Two immunoradiometric assays of VIII:CAg were developed with the use of different immune sera. In both assays the preparation of labelled antibodies from the sera and the assay techniques were identical.

IgG from the sera was isolated by Protein-A-Sepharose and labelled with ^{125}I . Specific, labelled antibodies were purified by complex formation in solution with a factor VIII concentrate (Hemofil, Hyland) and the complex was isolated by chromatography on Sepharose 6 B. The complex was dissociated and the specific labelled antibody obtained by chromatography on Sephadex G-200 at acid pH.

The assay was performed as a solid phase two-site technique as described for VIIIR:Ag (Page 14).

The first method, described in detail in Paper II, is based on two homologous antibodies spontaneously developed in non-haemophilic patients. The titres were 250 U/ml and 500 U/ml, respectively (one unit being the amount that inhibits VIII:C present in 1 ml normal, human, pooled plasma). The low-titre antibody was used for coating in the first step of the assay and the high-titre antibody was labelled and used in the third step. The limit of detection of VIII:CAG is 0.8 U/dl. Normal range is 40-150 U/dl.

The second method, described in detail in Paper IV, is based on a homologous antibody developed in a haemophilic patient. The titre was 1000 U/ml. The limit of detection is 0.5 U/dl and the normal range 40-150 U/dl. The inter-assay coefficient of variation is 9% at a concentration of 35 U/dl.

Discussion

The preparation of VIII:C, devoid of VIIIR:Ag and VIIIR:RCF, clearly indicates that the two components are separate entities. The fact that the VIII:C preparations could be activated by thrombin makes it probable that they contain a protein which is, at least partially, native. Recent

studies demonstrating that thrombin activates VIII:C by a proteolytic action supports this view (31, 70). The VIII:C protein was not obtained in an amount sufficient to allow biochemical characterization or immunization of rabbits.

The specificity of the rabbit antibody for VIIIIR:Ag, is clearly demonstrated by its ability to block VIIIIR:Ag, but not to neutralize VIII:C when mixed with normal plasma. When used in the IRMA it does not detect any protein in plasmas from patients with severe von Willebrand's disease. The complex formation between VIII:C and VIIIIR:Ag is shown by the fact that when immobilized on a matrix the antibody extracts both from plasma.

The specificity of the homologous antibodies to VIII:C was demonstrated by the good agreement between VIII:C and VIII:CAg in normal plasma and in plasmas from severe haemophiliacs. In the plasmas from patients with severe von Willebrand's disease, small, though equal, amounts of VIII:C and VIII:CAg were found, but no VIIIIR:Ag. These findings have been made independently by us and by others (71, 72, 80), who have developed similar immunoradiometric assays of VIII:CAg. Normal serum, although devoid of VIII:C, contains VIII:CAg, but somewhat less than plasma, suggesting that only a limited degree of proteolysis occurs in VIII:CAg during the process of coagulation (31).

Theoretically, the specificity of the VIII:CAg assay should be better with the use of two different antibodies, one for coating and one for labelling, as in Paper II. As described below (page 21), however, good agreement was found between the results obtained with this method and the one based on only a single antibody (Paper IV).

GENETIC VARIATION IN HAEMOPHILIA

Haemophilia A - Papers II, III, IV

Background

Haemophilia A is classified as severe (<1%), moderate (1-4%) or mild (5-25%), according to the level of VIII:C, measured by various coagulation assays (6, 12). The most widely used assays are one-stage recalcification methods, in which the ability of a patient's plasma to correct the defect in severe haemophilia A is compared with that of normal plasma. The affected members of a family have a deficiency of the same severity (6). The underlying molecular abnormality responsible for the deficiency is not known. Table I shows the number of haemophilia A patients in Sweden.

Type	Severe	Moderate	Mild	Total
Number	136 (108)	71 (45)	248 (113)	455 (266)

Table I. The number of patients with haemophilia A, of varying severity in Sweden 1980 (number of families given in brackets).

The earliest efforts to investigate the molecular defect in haemophilia A utilized immunological techniques and rabbit antisera (24, 73, 74). It is now obvious that those antisera were directed mainly against what we now recognize as the von Willebrand factor. Heterologous antisera against that part of the factor VIII complex responsible for VIII:C proved difficult to produce. Therefore interest was focused early on homologous antibodies. These are of two different

origins. Haemophilic antibodies arise in about 10% of multitransfused severe haemophiliacs. Spontaneous antibodies can develop as an auto-immune phenomenon in non-haemophilic individuals and cause acquired haemophilia A. Human antibodies were found to be non-precipitating and were therefore first used in neutralization assays. Two genetically distinct types of haemophilia were identified with such assays (13, 15, 75). About 10% of haemophilia A plasmas were found to neutralize a homologous antibody to VIII:C. It was concluded that some haemophilia A plasmas had a biologically non-functional, but immunologically cross-reacting (CRM+) protein associated with VIII:C, while the majority lacked such cross-reacting material (CRM-). In subsequent studies, a varying proportion of CRM+ cases were found, but, with one exception (76), CRM+ was never seen in severe haemophilia A (77-79). Neutralization tests are difficult to perform with accuracy. They are less sensitive, their detection level is usually 10-25 U/dl, and they should be considered qualitative rather than quantitative.

The development of immunoradiometric assays, based on the same type of antibodies but radiolabelled and purified, has permitted a more precise analysis of the immunological properties of VIII:CAg in haemophilic plasmas (71, 72, Paper II). These studies immediately revealed a variation in the amount of VIII:CAg in patients with haemophilia A, but it was not clear whether this heterogeneity was genetically determined. This aspect, however, became increasingly important when the IRMAs began to be used for prenatal diagnosis of haemophilia A.

The purpose of this part of the investigation was to study the variation of VIII:CAg in haemophilia A of different types by two available IRMAs, and to ascertain whether there are genetically determined subgroups of the disease even within those types which are of equal severity. An

ultimate goal was to find out the applicability of IRMA:s in prenatal studies.

Results

a) Severe haemophilia A. In Paper II, VIII:CAG was measured in 13 patients (13 families) with severe haemophilia A. The IRMA for VIII:CAG, detailed in the same paper and utilizing two spontaneous antibodies was used. None of the patients had detectable amounts of VIII:CAG.

In paper IV the clinical material, by then increased to 15 patients (14 families), was reinvestigated with the use of two IRMA:s for VIII:CAG. None of the patients, except one - who had a small amount (1.6 U/dl) measured with the method based on spontaneous antibodies - had demonstrable VIII:CAG with any of the two methods.

b) Moderate and mild haemophilia A. In moderate and mild haemophilia A, three subtypes could be distinguished (Paper III). Type I had no detectable VIII:CAG, type IIa had VIII:CAG levels lower than, or approximately equal to, that of VIII:C, and type IIb had higher levels of VIII:CAG than VIII:C. They were regarded as being type IIa or IIb according to the ratio VIII:CAG/VIII:C (IIb: ratio >1.5). The distribution of the 54 (28 families) patients among the three subtypes is shown in Table II.

Classification according to VIII:C (U/dl)	Classification according to VIII:CAG		
	type I	type IIa	type IIb
Moderate (1-4)	10 (6)	3 (2)	7 (2)
Mild (5-25)	14 (7)	9 (5)	11 (6)

Table II. Classification of 54 moderate and mild haemophiliacs according to the level of VIII:CAG. The number of families is given in brackets.

There was no difference in VIII:C between the subgroups of the moderate or of the mild form of the disease. VIII:R:Ag were normal in all patients. Ivy bleeding time was normal in all patients. A homogenous pattern of VIII:CAg was found within each family where more than one affected member could be examined.

To find out whether the types found were dependent upon the antibody used, part of the material, with addition of some new families, were examined also with the IRMA based on the haemophilic antibody (Paper IV). In the moderate type of the disease (11 patients, 5 families) there was good agreement between VIII:CAg levels measured with the two methods. In the mild cases (21 patients, 14 families), two patients classified as type I with the haemophilic antibody, had small amounts of VIII:CAg detectable with the spontaneous antibodies.

Discussion

Patients with severe haemophilia A showed a very uniform pattern with no detectable VIII:CAg (CRM-), with the exception of one who had a tiny amount measured with one of the IRMAs. Similar results have been obtained by other investigators using solid phase two-site techniques (80-82). However, a slightly larger proportion of CRM+ cases have been reported by investigators using fluid phase one-site techniques (72, 83-85).

A two-site technique requires two immunoreactive sites on the antigen available for the antibodies, and this might explain the differences between results obtained with one-site and two-site techniques. The good agreement between the results obtained with the two IRMAs in severe haemophilia A favours the view that in this form of the disease there is a real lack of the protein

associated with VIII:C rather than a structural defect in its antigenic sites making it unrecognizable by the antibodies.

The types found in moderate and mild haemophilia A seem to be fairly independent of the antibodies used. Reisner et al (84) also found good agreement between the results obtained with five different alloantisera. Thus, variation in the assay technique seems to affect the results more than variation of the source of antibody.

In type I of moderate and mild haemophilia A, which lacks demonstrable VIII:CAg, a major immunoreactive site is probably missing in the molecule, which is thus not recognized in the assay. The immunoreactive site is probably remote from the sites active in coagulation, since the molecule retains some biological activity. Investigators using one-site fluid phase techniques have not found this type of moderate or mild haemophilia A (72, 83-85). The good agreement between the results obtained with the two IRMAs in type II plasmas suggests that there is a quantitative reduction of VIII:CAg in this type. In type IIb there must obviously be a qualitative defect, too. Some of the patients in type IIb had almost normal levels of VIII:CAg. At present it is not possible to separate this VIII:CAg from normal VIII:CAg.

More than one affected member could be examined in each of fifteen kindreds of haemophilia A with all together 42 members. Affected members of a family invariably had the same type of the disease, strongly indicating that the subtypes and the molecular variation found are genetically determined.

Family XVII in Paper III lends further support to this view. One of the two examined members of the family

with mild haemophilia A type I had developed an anti-coagulant against VIII:C. This is extremely rare; only 8 cases were known in the literature when reviewed by Shapiro (22). Recently also the other member of the family developed an anticoagulant against VIII:C. One must conclude that the protein which is associated with VIII:C in this family and which is not detectable by IRMA, is of a special kind and quite different from the normal protein since the latter is recognized as non-self and acts as a strong antigen when given as a factor VIII concentrate.

Haemophilia B (Paper V)

Background

Haemophilia B is clinically indistinguishable from haemophilia A and occurs in severe (IX:C <1%), moderate (IX:C 1-4%) and mild forms (IX:C 5-25%). The clinical severity does not differ between affected members of the same family, and thus indicates a genetic origin of the different forms of the disease (6, 86). Table III gives the number of haemophilia B patients in Sweden.

Type	Severe	Moderate	Mild	Total
Number	31 (23)	19 (12)	59 (29)	109 (64)

Table III. The number of patients with haemophilia B with varying severity in Sweden 1980 (number of families given in brackets).

Fantl et al (87) were the first to demonstrate immunological variants of factor IX by showing that a specific human antibody to IX:C was neutralized by plasma from some patients but not from others. By refined neutralization techniques haemophilia B plasmas could be classified as: CRM+ with normal amounts of IX:Ag but diminished coagulant activity (IX:C), as CRM_R with IX:Ag reduced to the roughly same degree as IX:C, and as CRM- in which IX:Ag was lacking or not cross-reacting with the antibody used (14, 88). These results were obtained by using homologous non-precipitating antibodies in neutralization tests. Such tests are less sensitive with a limit of detection down to only around 10-15 U/dl.

An important advance was the elaboration of more exact immunological methods, such as electroimmunoassay (89, 90), radioimmunoassay (91) and immunoradiometric assay (92), all based on heterologous antisera. Probably owing to differences in the specificity of the various antibodies used, and to the sensitivity limits of the assays, the proportions found between CRM+ and CRM- have been conflicting (89 - 98). Only few data are available on the molecular variation within and between families with haemophilia B, especially on families with IX:Ag below 10 U/dl.

The heterogeneity in haemophilia B is further increased by the existence of variants characterized mainly by a prolonged prothrombin time when the plasma is tested with bovine brain thromboplastin, but not with human brain thromboplastin (99, 100). Even this variant is heterogeneous since it includes both CRM+ and CRM- cases (77). Haemophilia B Leyden, described in some families with moderately severe haemophilia B, is characterized by a diminishing bleeding tendency and an increasing IX:C with increasing age (101).

The purpose of this part of the study was to shed light on the genetic variation in haemophilia B with the use of an immunoradiometric assay of IX:Ag (54), more sensitive than previous assays and capable of measuring IX:Ag in foetal blood, and to ascertain how applicable this assay is for prenatal diagnosis.

Results

Of the 18 investigated patients with severe haemophilia B, 13 had no demonstrable IX:Ag, 4 had very low levels and one a higher but still subnormal level. Of the 9 patients with moderately severe haemophilia B, 4 (3 families) had IX:Ag approximately equal in amount to IX:C and 5 (3 families) had IX:Ag much higher than IX:C but still in the sub-normal range. Of the 21 patients with mild haemophilia B, 11 (5 families) had IX:Ag approximately equal to IX:C and 3 (3 families) had normal levels. In families where more than one affected member could be tested all of them always had the same pattern, except in family No. 27.

All investigated patients had normal P & P, Thrombotest and one-stage prothrombin time tested with human and with bovine brain thromboplastin. A possible haemophilia B Leyden variant was found in one of the families with the mild form of the disease.

Discussion

Only one out of 18 patients (15 families) with severe haemophilia B was found to have a substantial amount of IX:Ag. This result differs somewhat from those of other investigators, who all used rabbit antisera (89, 91, 92, 96, 98). These authors found a larger proportion of cases with higher or even normal levels of IX:Ag. The differences between the present results and those of other investigators

might be attributed to differences in specificity of the immune sera. A homologous antibody, like the one used here, can be expected to react with fewer immunoreactive sites on the factor IX molecule than a heterologous antibody. Our solid phase two-site assay-technique cannot be the only explanation for the discrepancy, since a similar approach was used also by Yang (92).

Half of the moderate cases and the majority of the mild ones had IX:Ag approximately equal to IX:C. These cases probably had a reduced amount of factor IX protein. Those with a much higher level of IX:Ag than IX:C, or even a normal level, presumably had an abnormal molecule with well preserved immunological properties, but with greatly diminished functional ability.

Eight families (with all together 28 members) were available in whom more than one affected member could be examined. Members belonging to one and the same family always had the same level of IX:Ag, except in family 27, in which the disease might have been a haemophilia B Leyden variant, although never described before in mild haemophilia B.

A clinically important application of an IRMA is the prenatal evaluation of a fetus at risk for haemophilia B (see below). Obviously it is possible to distinguish affected fetuses from normal fetuses only in CRM- families, or in families with very low levels of IX:Ag. The IRMA used in this investigation and based on a homologous antibody with narrow specificity therefore has proved most useful, since it classifies almost all the severe families as CRM- and half of the moderate ones as CRM_R with a very low level of IX:Ag. Thus, prenatal diagnosis by IRMA would be possible in 80% of all families in whom it might be indicated.

PRENATAL DIAGNOSIS OF HAEMOPHILIA A AND B - Paper VIBackground

Genetic counselling in haemophilia has, until recently, been restricted to providing prospective parents with risk figures for their future offspring and an offer to determine the sex of the fetus. Faced with this information many female carriers have avoided pregnancy. Others have chosen to terminate pregnancies with male fetuses, a decision that entails a 50% risk of aborting a healthy fetus.

The improvement of the fetoscopic blood sampling technique has now enabled us to obtain fetal blood for examination in the 18th-20th gestational week (102). This provides us with the possibility of diagnosing inherited diseases expressed in the blood, but not in the amniotic fluid. The existence of VIII:C and VIIIR:Ag in fetuses at this gestational age is well established (103, 104).

A problem is to obtain a suitable pure anticoagulated fetal blood sample for analysis. A conventional coagulation analysis of VIII:C and IX:C requires addition of an exact amount of citrate to the fetal sample. Further, any dilution of the fetal plasma with amniotic fluid disturbs the assays since amniotic fluid contains thromboplastic substances which mimic VIII:C and IX:C. The development of immunoradiometric assays for VIII:CAg (71, 72, Paper II) and IX:Ag (54) has overcome this problem, since dilution with amniotic fluid does not influence determination of VIII:CAg and IX:Ag.

The aim of this part of the investigation was to summarize 2 years' experience of genetic counselling and prenatal diagnosis in haemophilia A and B.

Results

Thirty-one pregnant women, obligate or probable carriers of haemophilia A, requested sex-determination of the fetus and, in case of a male fetus, prenatal diagnosis by fetal blood sampling. Two women had spontaneous abortions, one before sex-determination and the other 3 weeks after an attempt to obtain fetal blood via the fetoscope had failed. In 2 cases the affected family had a genetic variant that prevented evaluation of the fetus. Sex-determination by amniocentesis in the 15th-16th gestational week showed female fetuses in 11 cases. The remaining 16 male fetuses were evaluated by immunoradiometric analysis of the blood samples obtained in the 18th-20th gestational week. Eleven fetuses were judged as unaffected and 5 as affected. In the latter group the pregnancy was interrupted. Blood obtained from the children post-partum and from the fetuses after abortion confirmed the prenatal diagnosis.

Three fetuses at risk for haemophilia B were examined in a similar way and all were found to be affected. The pregnancies were interrupted and the diagnosis was confirmed in 2 cases, while no blood was obtained from the third.

One third of the fetal samples were undoubtedly contaminated with amniotic fluid, and in the remaining cases a slight contamination could not be excluded with certainty. It was possible to calculate the dilution factor by determination of EVF (erythrocyte volume fraction) or erythrocyte count in the fetal samples, since normal values for these in fetuses are known (105). In haemophilia A, determination of VIIIIR:Ag and ratio VIII:CAg/VIIIIR:Ag, was found to be a useful internal standard in diluted samples.

Discussion

The frequent dilution of the fetal blood samples with amniotic fluid demonstrates the importance of using immunoradiometric assays for evaluation of factors VIII and IX. However, fetal blood samples should not be diluted more than 5 times, which leaves a good safety margin for the sensitivity of the IRMAs without risk of overdiagnosis.

The genetic variation in both haemophilia A and B poses a problem though when immunoradiometric assays are used. As shown earlier in this investigation, some families of moderate haemophilia A and severe and moderate haemophilia B have substantial amounts of respectively VIII:CAg and IX:Ag. In such families it is impossible to differentiate between a healthy and an affected fetus with the use of IRMAs. This was the case in 2 of the 31 pregnant women who requested prenatal diagnosis. The ideal situation would be one in which prenatal diagnosis of haemophilia is based on determinations of both VIII:C and VIII:CAg or IX:C and IX:Ag (106). In addition, future studies may find antisera with the ability to discriminate a normal VIII:CAg and IX:Ag from a functionally abnormal one, and thus make it possible to use IRMA in all instances when prenatal evaluation is indicated.

Another problem requiring future research is whether a possible carrier of haemophilia really is a carrier (16, 107). Eleven of the 16 investigated fetuses at risk for haemophilia A were found to be healthy, which may indicate that all the pregnant women were probably not true carriers.

In our experience prenatal diagnosis of haemophilia has proved so reliable that it should be included in genetic counselling in severe and moderate haemophilia. Especially

since both in our and other investigators' (106, 108) experience the majority of fetuses examined have been found to be healthy probably due to the difficulty in ascertaining the carrier status. This clearly shows how unsatisfactory it is to terminate all pregnancies with male fetuses.

IMMUNORADIOMETRIC DETECTION OF INHIBITORS IN HAEMOPHILIA A

Paper VII

Background

Inhibitors or anticoagulants against VIII:C have been found in about 10-20% of severe haemophilia A patients who have received substitution therapy (22). Inhibitors of VIII:C can also arise in non-haemophiliacs; e.g. previously healthy women after delivery, patients with various immunologic disorders and even persons with no co-existent disease. The inhibitory action is directed specifically against VIII:C. Haemophiliacs with inhibitors have normal VIIIIR:Ag, VIIIIR:RCF and bleeding time (22).

The inhibitors in haemophilia patients are IgG-antibodies with a single type of light chain, usually kappa, and a clear predominance of heavy chain subclass IgG:4 (109-111). Several antibodies examined have been exclusively IgG:4 (111, 112). The antibodies are non-precipitating and do not fix complement (22).

Practically only patients with severe haemophilia A develop inhibitors, although a few examples have been described in mild haemophilia A (22). It has been hypothesised that CRM- patients are more prone to recognise a given Factor VIII preparation as non-self and elicit an immune response (113). Since most severe haemophilia A patients are CRM- and only some develop antibodies, other factors must be involved. It seems that a genetic predisposition plays a part (114), although no clustering was found within families in a large material investigated by Biggs (115). The introduction of prophylactic treatment has not increased the number of patients with inhibitors (116).

Regular examination for anticoagulants with sensitive tests is important in the care of haemophiliacs. The methods hitherto used have been neutralization tests, which exist in several modifications (52, 53, 117). Such tests are subject to methodological difficulties, the results are difficult to interpret and the lowest inhibitor-titre that can be measured with certainty is usually around 0.5 U/dl.

The purpose of this part of the investigation was to develop a more sensitive and precise immunoradiometric assay for the detection of inhibitors of VIII:C, and to apply this assay to patients in whom the existence of such inhibitors was doubtful.

Results

The assay, detailed in Paper VII, is based on the principle of competition for VIII:CAg between labelled antibodies and antibodies in a test sample. For this purpose VIII:CAg was immobilized by antibodies to a solid phase. A reference curve was constructed with the inhibitory plasma, from which the labelled antibody had been purified, and used to quantify inhibitor in other inhibitory plasmas (Fig. I). The reference plasma was arbitrarily said to have 1000 inhibitory U/ml, since this was the titre obtained in the neutralization test. The lowest titre that could be measured was 0.02 U/ml.

Fifteen haemophiliacs who were known to have or have had inhibitors to VIII:C were tested with a neutralization assay and the IRMA. In three cases the antibody was detected only by the IRMA. In the other patients there was rather good agreement between the values obtained by the two methods. The dose-response curves for all the examined antibodies were parallel to the reference curve.

Three non-haemophilic patients with inhibitors were tested and a parallel dose-response curve was found in 2, but not in the third (Fig. I).

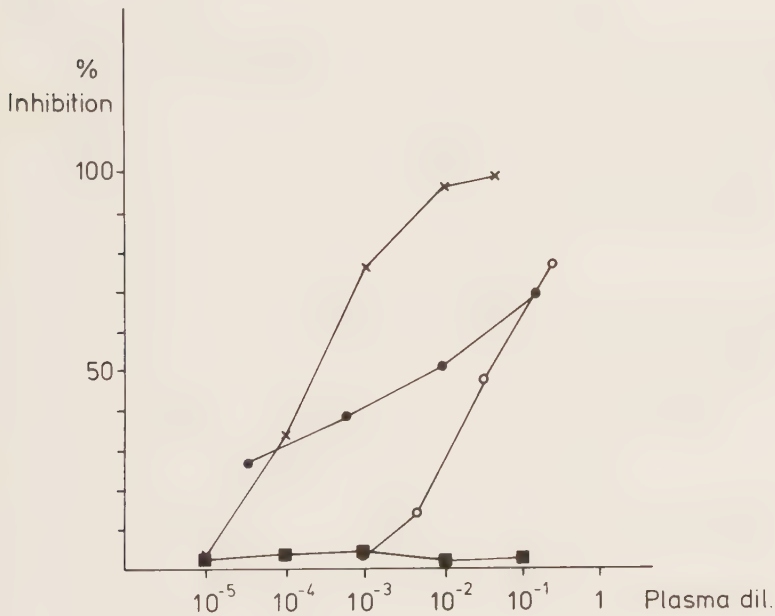


Fig. I. Inhibitor curves obtained for the reference plasma (X-X), normal IgG (■-■) and IgG from one haemophiliac (O-O) and one non haemophiliac (●-●) with inhibitors.

Discussion

The reference antibody used in the assay seems to have an affinity for VIII:CAg which is common to all the haemophilic antibodies tested, since parallel inhibition curves were obtained in all the cases. Still, it is conceivable that different antibodies react at different sites on the VIII:C molecule. Some might be directed against antigenic determinants close to the coagulation active site

and others to antigenic sites remote from it, so that such antibodies may not be detectable by a neutralization assay but only with an assay based on competitive binding of antibodies.

The antibody used in the IRMA seems to be most appropriate since it competes with all the inhibitors found by the neutralization test. The amounts of inhibitor estimated with the two methods showed good agreement. The same observation has been made by Furlong et al (118), who used a similar method.

The new assay also detected the three non-haemophilic antibodies examined. One gave a non-parallel dose-response curve suggesting a different affinity.

The IRMA is about 20 times as sensitive as the neutralization assay and thus of practical value in the investigation of cases with low inhibitor titres and perhaps also for early detection of a developing anticoagulant.

It can be hypothesised that haemophiliacs on prophylactic treatment, who gradually require increasing doses to obtain a certain level of VIII:C, or who have a short $T_{\frac{1}{2}}$ of infused Factor VIII might have weak, non-neutralizing antibodies. The IRMA based on competition between antibodies for the same antigen, may perhaps be able to reveal such antibodies.

CONCLUSIONS

- 1 a It is possible to separate antihaemophilic factor A (VIII:C) from the von Willebrand factor (VIII:RAg). Antigenic determinants of the von Willebrand factor are not detected in the VIII:C preparation even by a very sensitive IRMA for VIII:RAg, which indicates that they are separate entities.
- 1 b Specific homologous antibodies, haemophilic or spontaneous, can be labelled, purified and used in the development of immunoradiometric assays that are specific for VIII:C (VIII:CAg) and that do not detect VIII:RAg.
- 2 a There are several genetic variants of haemophilia A. In the severe form of the disease, VIII:CAg is almost invariably lacking. Moderate as well as mild haemophilia A can be classified into three genetically determined subtypes according to the content of VIII:CAg. Type I has no demonstrable VIII:CAg, type IIa has VIII:CAg in an amount smaller than, or approximately equal to, that of VIII:C and type IIb has a larger amount.
- 2 b According to the immunoradiometric assay, most, but not all, patients with severe haemophilia B lack demonstrable IX:Ag. In the moderate and mild forms, IX:Ag is demonstrable either in amounts equal to IX:C or in larger or even normal amounts.

- 3 Immunoradiometric assays of VIII:CAg and IX:Ag are suitable for prenatal diagnosis in at least 90% of all cases of haemophilia A and 80% of haemophilia B, in which evaluation of a fetus at risk for haemophilia is indicated. Prenatal diagnosis of haemophilia A and B has proved reliable and should be included in the genetic counselling for the severe and moderate forms of these diseases.
- 4 Anticoagulant detection in haemophilia A can be greatly improved by the use of a sensitive immunoradiometric assay. The possibility of revealing antibodies with various specificities is improved.

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PURIFICATION OF F.VIII:C BY ANTIGEN-ANTIBODY CHROMATOGRAPHY

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ABSTRACT

Purification of F.VIII:C devoid of F.VIII:Ag was achieved by antigen-antibody chromatography. The antibody used neutralized VIII:Ag but not VIII:C in liquid phase but extracted both VIII:Ag and VIII:C from plasma when bound to Sepharose. VIII:C was eluted with calcium-containing buffer. When plasma was used as starting material VIII:C was obtained free from VIII:Ag but contaminated with some other proteins. When a well-defined pure F.VIII preparation was used as starting material the VIII:C active fractions contained no immunoradiometrically detectable VIII:Ag, no VIII:RCF and no detectable protein. When stabilized with albumin VIII:C could be frozen and thawed with retained activity and could be activated with thrombin.

INTRODUCTION

Factor VIII clotting activity (VIII:C) elutes together with factor VIII related antigen (VIII:Ag) at physiological ionic strength. By gel filtration at high salt concentrations or under mild reducing conditions it has been possible to separate VIII:C from VIII:Ag more or less completely (1,2,3,4). However, it has been questioned whether this separate VIII:C represents a native protein but rather is a proteolytic product of VIII:Ag (5,6). This paper reports the preparation of VIII:C devoid of any immunoradiometrically detectable VIII:Ag using an immobilized antibody to VIII:Ag.

MATERIAL AND METHODS

Preparation of antiserum to VIII:Ag. The contents of one vial of Hemo-fil (Hyland), which was outdated for clinical use, were dissolved in 15 ml water and chromatographed on Sepharose 6 B according to van Mourik and Mochtar (7). Rabbits were injected every fortnight with 1 ml of the void volume peak, emulsified with 1 ml Freund's complete adjuvant. A high antibody titre was obtained after 4 injections. After absorption with plasma from a patient with severe von Willebrand's disease the antiserum gave one line with normal plasma but none with von Willebrand's disease plasma in double diffusion. The antiserum had a very slight inhibitory effect on VIII:C when mixed with plasma (Table 1). IgG from the antiserum was prepared according to Steinbuch and Audran (8).

TABLE 1
Effect of antiserum on plasma VIII:C and VIII:Ag

Mixture	F.VIII:C U/ml	F.VIII:Ag U/ml
Control plasma (dil. 4/5)	0.79	0.91
Antiserum + plasma 1/1000	0.55	0.56
1/500	0.47	0.30
1/200	0.60	0
1/100	0.45	0
1/50	0.47	0
1/10	0.55	0
1/5	0.48	0

The antiserum was heated to 56°C for 30 min., adsorbed with BaSO₄ and incubated with plasma for 1 hour in various proportions.

Preparation of affinity column. 15 g CNBr-activated Sepharose 6 B was reacted with 310 mg antiserum IgG at pH 8.3. After washing the gel was treated with 2 % Blue dextran (Pharmacia) in carbonate buffer, pH 9, for 2 hours. After further washing at pH 4.0 and pH 8.0 until the absorbance was zero the gel was packed in a 1.6 x 40 cm column in 0.05 M Tris-HCl buffer, 0.15 M NaCl, pH 7.4. In the experiments using plasma the starting buffer contained 0.2 % trisodium citrate, which was changed to citrate-free buffer when all the plasma had entered the column. VIII:C was eluted with 0.05 M Tris-HCl buffer, 0.25 M CaCl₂, pH 7.4. The column was regenerated by washing with 3 M NaSCN in 0.1 M citrate buffer, pH 6.0 and then equilibrated with the starting buffer. It could be used several times.

Purification of factor VIII. This was purified by gel chromatography of

2 units cryoprecipitate (prepared fresh from 400 ml plasma) on Sepharose 6 B, equilibrated with 3 % dextran according to van Mourik and Mochtar (7).

SDS-polyacrylamide gel electrophoresis was performed according to Weber and Osborn (9).

VIII:C was determined in a one-stage recalcification system (10). Samples were always tested diluted 1:100 or more to eliminate errors due to the calcium content in the samples. In the thrombin activation experiments all dilutions were made in 0.05 M Tris-HCl, 0.15 M NaCl, 2 % BSA, pH 7.4.

Activation with thrombin. Human plasminogen-free thrombin (KABI DcT 7B) was dissolved in 0.05 M Tris-HCl, 0.15 M NaCl, 2 % BSA, pH 7.4 to a concentration of 0.02 U/ml. The thrombin solution was added to an equal volume of dissociated VIII:C and incubated at +23°C. VIII:C was determined at 10, 30, 60 and 120 min. The following controls were used: 1) the thrombin solution + an equal volume 0.05 M Tris-HCl, 0.25 M CaCl₂, pH 7.4, 2) VIII:C + an equal volume 0.05 M Tris-HCl, 0.15 M NaCl, 2 % BSA, pH 7.4.

Unspecific thromboplastic activity was revealed by testing the fractions not only for VIII:C but also for their ability to normalize haemophilia B plasma in a one-stage recalcification system.

VIII:R:Ag was determined in 2 ways: 1) Quantitative electroimmunoassay (11), 2) Immunoradiometric assay essentially as described by Ruggeri et al (12). The antiserum described in Table 1 was used. 0.36 mg antiserum IgG was labelled with 2 mCi ¹²⁵I with the lactoperoxidase method. Unreacted material was removed by gel filtration on Sephadex G-50 in 0.04 M phosphate buffer, 0.1 M NaCl, 1 % BSA, pH 7.4. The labelled IgG (in 1 ml) was incubated for 1 hour at +37°C with 1 ml factor VIII concentrate (AHF KABI). The incubation mixture was diluted to 4 ml and chromatographed on a 1.6 x 60 cm column of Sepharose 6 B in 0.04 M phosphate buffer, 0.1 M NaCl, 0.25 % BSA, pH 7.4, flow rate 8 ml/hour. A peak of radioactivity, amounting to 2.2 % of the total radioactivity, was eluted at the void volume. This peak (5 ml) was dialyzed over night against 0.05 M glycine-HCl buffer, 0.1 M NaCl, pH 2.4 and then chromatographed on a 2.6 x 75 cm column of Sephadex G-200 in the same buffer but with 1 % BSA, flow rate 7.5 ml/hour. Eighty per cent of the radioactivity eluted in a sharp peak coinciding with the elution volume of IgG and this peak was used. The test was performed in the following way: 55 x 11 mm polystyrene tubes (Ellerman tubes, Medart AB, Sweden) were coated with antibody to VIII:R:Ag by incubation with 0.6 ml of an antiserum dilution 1:5000 in 0.05 M carbonate buffer, pH 9.6 for 12 hours at +4°C. The tubes were washed 3 times with PBS, 0.1 % BSA. The samples to be tested, diluted to 0.4 ml in

0.05 M Tris-HCl, 0.1 M NaCl, 6 % BSA, pH 7.4 were then incubated in the tubes for 12 hours at $+37^{\circ}\text{C}$. After washing twice with PBS, 0.1 % BSA, 0.4 ml labeled antibody solution, containing about 8000 cpm, was added and the tubes were again incubated for 12 hours at $+37^{\circ}\text{C}$. After washing in water the tubes were counted for 5 min. in a gamma scintillator. The dose-response curve of normal plasma is shown in FIG. 3. The limit of detection of VIIIR:Ag is $0.5 \cdot 10^{-4}$ U/ml. Patients with severe classical von Willebrand's disease had VIIIR:Ag below this limit.

Ristocetin cofactor activity (VIIIR:RCF) was quantitated using formal fixed platelets (13). The standard curve was constructed by mixing normal plasma with plasma devoid of VIIIR:RCF (from a patient with severe von Willebrand's disease) in various proportions to a final volume of 0.5 ml, adding 0.1 ml ristocetin solution (Lundbeck, Copenhagen), and 0.4 ml of platelet suspension and recording the aggregation velocities in an aggregometer. The maximal slope of the aggregation curve was plotted against the concentration of test plasma on a lin/log scale. The fractions to be tested were dialyzed against 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.4, and 0.1 ml was mixed with 0.4 ml of the von Willebrand's disease plasma and the samples were then handled in the same way as the standard.

RESULTS

The separation of VIII:C from VIIIR:Ag by affinity chromatography of plasma is shown in FIG. 1. Eighty ml of fresh citrated plasma was applied to the column at $+4^{\circ}\text{C}$. The effluent was monitored for protein at 280 nm and tested for VIII:C and VIIIR:Ag. A small amount of VIIIR:Ag, about 4 % of that applied, passed the column without being bound. The column was washed extensively until absorption at 280 nm reached a plateau. A small protein peak was then eluted with buffer containing 0.25 M/l CaCl_2 . In the peak 50 % of the VIII:C applied was recovered. However, the fraction also shortened the recalcification time of haemophilia B plasma, although to a much less extent (the unspecific F.IX-like activity was calculated to about 5 % of VIII:C). No VIIIR:Ag (tested with electroimmunoassay) or VIIIR:RCF were detected in the VIII:C active fractions. When the column was washed with 3 M NaSCN a large protein peak appeared, which, however, also had no detectable VIIIR:Ag, probably because of destruction of the antigenic structures by the washing procedure. VIII:C of the active preparation was moderately stable. After one week at $+4^{\circ}\text{C}$ the activity had decreased to half the original.

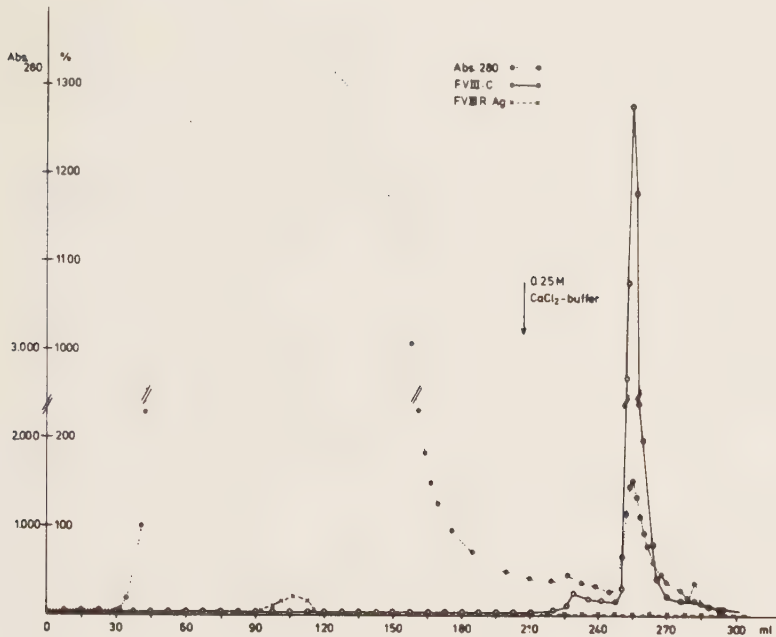


FIG. 1
Separation of VIII:C from VIII:R Ag by affinity chromatography of plasma.

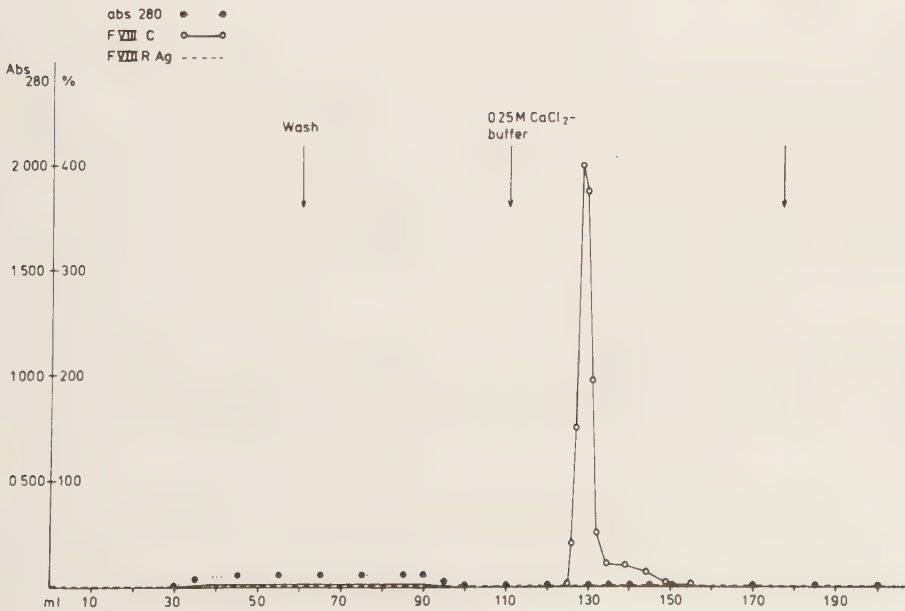


FIG. 2
Purification of VIII:C from a F.VIII preparation by affinity chromatography.

The experiment was then performed with a pure factor VIII preparation as starting material instead of plasma. Pure factor VIII was obtained by gel chromatography of fresh cryoprecipitate on Sepharose 6 B as described in Methods. Fifty ml of the pure factor VIII preparation contained 60 units VIIIR:Ag, 48 units VIII:C and about 1.2 mg protein. On SDS-polyacrylamide gel electrophoresis after reduction it gave one band with a molecular weight of about 200 000. The material was immediately applied to the affinity column at +4°C, 8 ml/hour (FIG. 2).

No VIIIR:Ag or VIII:C passed the column. The absorbance at 280 nm in the effluent was due to the dextran in the preparation. When the factor VIII containing material had entered the column it was washed with 1-2 column volumes of buffer. The buffer was then changed to 0.05 M Tris-HCl, 0.25 M CaCl₂, pH 7.4. VIII:C was eluted by the calcium-containing buffer in 4-5 fractions of 1.2 ml containing no detectable protein, measured as A₂₈₀. Bovine serum albumin was added to make a final 1 % solution. The fractions could then be frozen, stored at -60°C and thawed with retained activity. The VIII:C active fractions did not shorten the recalcification time of haemophilia B plasma.

The recovery of VIII:C in the affinity chromatography step was 34 %. When calculated relative to the VIII:C in the cryoprecipitate used for Sepharose chromatography it was 10 % (Table 2).

TABLE 2

	F.VIII:C	Protein
Cryoprecipitate	185 U (100 %)	925 mg
Sepharose chrom. material	52 U (28 %)	1.2 mg
Affinity chrom. material	17.8 U (10 %)	Not detectable
Recovery of Factor VIII:C		

The VIII:C active fractions were tested for VIIIR:Ag both with electroimmunoassay and immunoradiometry. No peaks were present in the electroimmunoassay. The results of the immunoradiometric assays are shown in FIG. 3. The amount of VIIIR:Ag was lower than 10⁻⁴ U/ml in fractions where VIII:C was 4 U/ml.

VIIIR:RCF could not be detected in the VIII:C active fractions.

The activation studies with thrombin are shown in FIG. 4. When incubated with human thrombin in a concentration of 0.01 U/ml there was a slight but clear increase of activity to about twice the starting level.

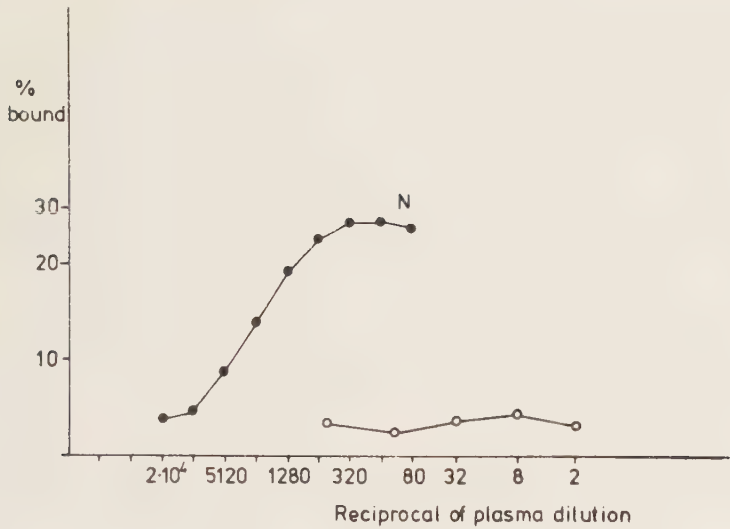


FIG. 3

Immunoradiometric assay of VIII:Ag. Dose-response curves of normal plasma (N) and VIII:C, purified by antigen-antibody chromatography. In purified VIII:C VIII:Ag was below the detection limit of the method.

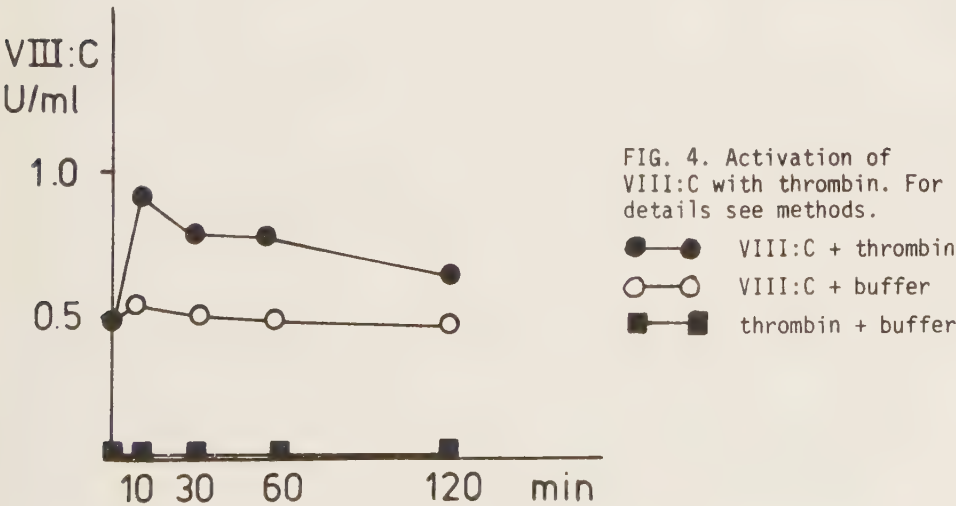


FIG. 4. Activation of VIII:C with thrombin. For details see methods.

DISCUSSION

Several workers have shown that it is possible to alter the elution pattern of VIII:C in gel filtration by increasing the ionic strength. In this way VIII:C can be more or less separated from VIII:Ag and VIII:RCF or a platelet aggregation factor (2,14). The molecular weight of VIII:C has been

variously estimated on different gels, from 25 000 to 240 000 Daltons. All workers, however, have used crude factor VIII preparations and it cannot be excluded that these preparations are contaminated with proteolytic enzymes (5).

The advantage with the method described here is that a well-defined and highly purified factor VIII preparation could be used for the dissociation experiments, minimizing the risk of having proteolytic contaminants. With an affinity column the pure factor VIII preparation could be used without prior concentration. Concentration of such pure factor VIII preparations is not possible to achieve without great loss of activity. The method could not be used with plasma as starting material, as there was too much unspecific absorption of other proteins in the column. When plasma was used VIII:C active fractions also slightly shortened the recalcification time of haemophilia B plasma and thus seem to be contaminated with an unspecific thromboplastic substance (FIG. 1). Therefore an antecedent purification step was necessary.

The present method is based on an antibody to factor VIII with quite remarkable properties. A similar antibody has been described before (15). The antibody was produced by immunizing rabbits with a pure factor VIII obtained from an aged and no doubt somewhat denatured factor VIII preparation. When bound to Sepharose the antibody removed both VIIIR:Ag and VIII:C from plasma or a factor VIII preparation. When mixed with plasma the antiserum completely neutralized VIIIR:Ag but VIII:C was only little affected. This is consistent with the hypothesis that VIIIR:Ag and VIII:C are separate entities, which, however, circulate in plasma complexed to each other and that antibodies can selectively be directed against one or the other of the components of the complex.

The present work shows that it is possible to obtain VIII:C completely devoid of VIIIR:Ag from a pure factor VIII preparation without contaminants detectable in SDS-polyacrylamide gel electrophoresis. The VIII:C could also be activated with thrombin (16). Thus it is probable that the VIII:C obtained derives from a native, non-degraded protein.

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Measurement of Antihaemophilic Factor A Antigen (VIII:CAg) with a Solid Phase Immunoradiometric Method Based on Homologous Non-Haemophilic Antibodies

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Antihaemophilic-factor-A-antibodies, which had spontaneously arisen in 2 patients, were used to develop an immunoradiometric method for measurement of antihaemophilic factor A antigen (VIII:CAg). 13 patients with severe haemophilia A had VIII:CAg below the limit of detection (0.01 U/ml). Patients with moderate and mild haemophilia A either had VIII:CAg roughly equal to factor VIII clotting activity (VIII:C) or a not detectable VIII:CAg, suggesting 2 different molecular mechanisms in moderate and mild haemophilia A. VIII:CAg could be detected in serum but in lower amounts than in plasma. In 2 patients with von Willebrand's disease VIII:CAg equalled VIII:C. The post-transfusional retarded increase of VIII:C in 1 patient with von Willebrand's disease was accompanied by a slight increase in VIII:CAg. Fetal plasma contained measurable amounts of VIII:CAg.

Key words: factor VIII – haemophilia A – immunoradiometric assay

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Congenital diseases with low factor VIII procoagulant activity (VIII:C) include classical X-linked haemophilia A and autosomal von Willebrand's disease. Immunological studies with heterologous antibodies have shown that in the later there is a deficiency of a plasma protein associated with VIII:C and called factor VIII related antigen (VIIR:Ag) (Zimmerman et al 1971, Holmberg & Nilsson 1972, Peake et al 1974). Immunological methods are there-

fore now important in the diagnosis of von Willebrand's disease. In haemophilia A, however, VIIR:Ag is normal or even increased (Zimmerman et al 1971, Holmberg & Nilsson 1973), and the diagnosis has been based solely on the demonstration of low factor VIII procoagulant activity (VIII:C) as measured in conventional clotting assays. Specific heterologous antisera against the unknown molecular structure responsible for VIII:C activity have proved dif-

ficult to prepare. However, homologous antibodies to VIII:C can arise in haemophiliacs after repeated infusions, in patients with autoimmune and myeloproliferative diseases; and also spontaneously (Shapiro & Hultin 1975). Such antibodies have been used in neutralization assays, but the question whether haemophiliacs lack specific antigens (VIII:Cag = VIII:C antigen) associated with VIII:C was not settled by such assays (Hoyer & Breckenridge 1968, Feinstein 1969, Denson et al 1969, Lechner 1972, Holmberg & Nilsson 1973, Biggs 1974, Zimmerman et al 1977, Poon & Ratnoff 1977). Recently Peake & Bloom (1978) and Lazarchick & Hoyer (1978) described immunoradiometric assays for VIII:Cag. We have developed a sensitive solid phase immunoradiometric method based on homologous non-haemophilic antibodies to VIII:C. This paper reports the results obtained with this assay in haemophilia A and von Willebrand's disease. VIII:Cag was determined also after infusion of factor VIII concentrates in patients with von Willebrand's disease. Also the VIII:Cag content of fetal plasma was studied.

METHODS

VIII:Cag. Sera from 2 non-haemophilic patients were used as sources of antibody. Both patients had spontaneously developed high titers of an inhibitor of VIII:C, but not of VIII:R:Ag or any other coagulation factor. The first patient was a man, aged 72 years, whose antibody has been known for half a year. The titre was 500 U/ml (Holmberg & Nilsson 1972) (1 unit being the amount of antibody capable of inhibiting an amount of VIII:C equal to that present in 1 ml of normal plasma). The second patient was a woman, aged 69 years, who has had an antibody against VIII:C for 8 years. Her titre at present is 250 U/ml.

IgG from the antisera was isolated by Protein A-Sepharose (Pharmacia Fine Chemicals) chroma-

tography. 5 ml of antiserum was applied to a 5 ml column. The column was washed with 0.05 M tris-HCl buffer, pH 7.4, 0.5 M NaCl, the IgG was eluted with 0.1 M glycine-HCl buffer, pH 3.0 and dialyzed against 0.04 M phosphate buffer, 0.1 M NaCl, pH 7.4.

The IgG from the first patient was used for labelling. 50 μ l of a preparation containing 10 mg/ml IgG and 400 U/ml inhibitor was labelled with 74 MBq 125 I with the lactoperoxidase method (Thorell & Johansson 1971). Unreacted material was removed by gel filtration on Sephadex G-50 fine in 0.04 M phosphate buffer, 0.1 M NaCl, 1 % bovine serum albumin (RIA grade, Sigma), pH 7.4. 90 % of the radioactivity was eluted with the IgG, corresponding to a specific activity of 133 MBq/mg. The inhibitory capacity of the IgG was not appreciably decreased by the labelling procedure. The labelled IgG (in 1 ml) was incubated for 2 h at +37°C with 1½ ml of factor VIII concentrate (Hemofil, Hyland, 25 U VIII:C/ml) and the incubation mixture chromatographed on a 1.6 $\times$ 60 cm column of Sepharose 6B in 0.04 M phosphate buffer, 0.1 M NaCl, 0.25 % BSA, pH 7.4. A small peak of radioactivity, amounting to 1.5 % of the total radioactivity, was eluted at the void volume. BSA to 1 % was added to this peak fraction (8 ml), which was dialyzed for 5 h against 0.05 M glycine-HCl buffer, 0.1 M NaCl, pH 2.4 and then chromatographed on a 2.6 $\times$ 75 cm column of Sephadex G-200 in the same buffer with 1 % of BSA. 60 % of the radioactivity was eluted in a peak coinciding with the elution volume of IgG and this peak was used after dialysis to neutral pH. It contained 40 % of the anti-VIII:C activity in the IgG used for labelling. IgG from the second patient was treated in the same way, but the yield of labelled antibody was less than 10 times that from the first patient.

The test was performed in the following way: 55 $\times$ 11 mm polystyrene tubes (Ellerman tubes, Medart AB, Sweden) were coated with IgG from the second patient by incubation with 0.6 ml of a dilution 1:100 in 0.1 M carbonate buffer, pH 9.6, for 12 h at +4°C. The tubes were washed 3 times with 1 ml of PBS, 0.1 % BSA. The samples to be tested in various dilutions with 0.01 M phosphate buffer, 0.1 M NaCl, 6 % BSA, pH 7.4, and in a total volume of 0.4 ml were then incubated in the tubes for 12 h at +37°C. The tubes were

again washed 3 times with PBS, 0.1 % BSA, and 0.4 ml labelled antibody solution was added. The labelled antibody was diluted 10–15 times with 0.01 M phosphate buffer, 0.1 M NaCl, containing 0.1 % of BSA, 0.05 % normal IgG (Kabi), to about 8000 cpm. After re-incubation for 12 h at $+37^{\circ}\text{C}$ and washing 3 times with 1 ml of water the tubes were counted for 10 min in a gamma scintillator.

VIII:Ag was determined with electroimmunoassay (Zimmerman et al 1971) or immunoradiometric assay (Ruggeri et al 1976). *VIII:C* was determined with a one-stage assay (Nilsson et al 1961). *VIII:RCF* was determined with formalin-fixed platelets as described by Zuzel et al (1978).

CLINICAL MATERIAL

Citrated plasma was obtained from 5 normal volunteers, 13 patients with severe haemophilia A, 4 with moderate haemophilia A, 4 with mild haemophilia A, and 2 with severe von Willebrand's disease. None of the haemophiliacs had received any f VIII infusion for at least 2 weeks.

1 patient with severe von Willebrand's disease got an infusion of 1500 U AHF Kabi and blood was drawn before and $\frac{1}{4}$, 1, 3, 5, 24 and 29 h after the infusion. Serum from the 5 normal persons was obtained by letting whole blood stand for 2 h at room temp. Blood from 4 fetuses (gestational age 17–20 weeks) was obtained at legal abortions performed by hysterotomy. One of the umbilical vessels was catheterized and the blood drawn immediately transferred to plastic tubes with 1 part of 3.8 % sodium citrate to 9 parts of blood.

RESULTS

A typical dose-response curve was obtained when normal plasma was tested in dilutions $1/4$ – $1/256$. The curve was linear in the region of $1/16$ to $1/128$ (Figure 1). The maximal amount of bound radioactivity was about 9 % and the background, obtained with buffer alone, was 1–1.5 % of the added radioactivity. The plasma dilu-

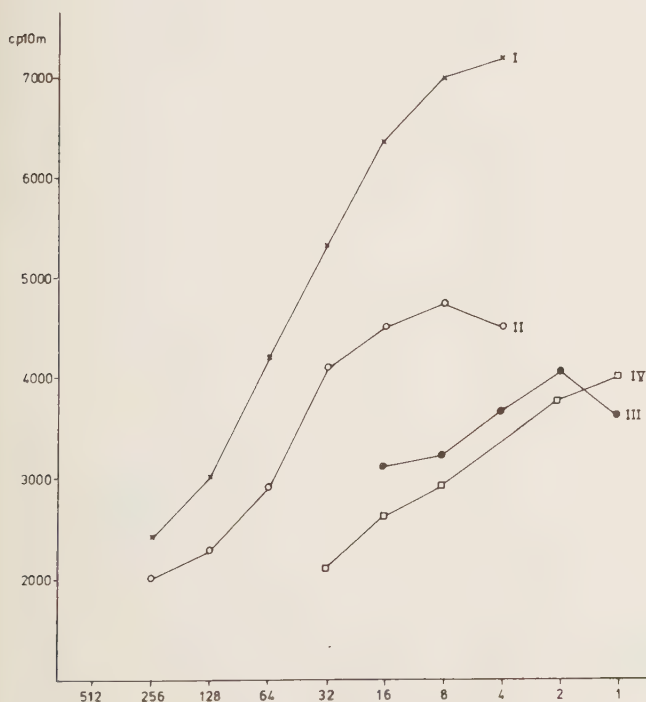


Figure 1. Dose-response curves. Reciprocals of plasma dilutions are plotted against cp 10 min. I: normal plasma, II: normal serum, III: 15 (moderate haemophilia A), IV: 14 (moderate haemophilia A).

TABLE 1
Factor VIII in plasma and serum from 5 normal persons

VIII:C (U/ml)	VIII:CAg (U/ml)	VIII:CAg (serum) (U/ml)	VIIIR:Ag (U/ml)
0.92	0.83	0.16	0.87
0.67	0.40	0.14	0.66
1.05	0.85	0.23	1.00
0.74	0.50	0.23	0.93
1.05	0.95	0.35	0.99

tion 1/128 gave twice the background level.

The results of the determination in 5 normal persons are given in Table 1. There was good agreement between determinations of VIII:C and VIII:CAg. VIII:CAg in the respective sera was well detectable but the levels were clearly lower than those in plas-

ma. The dose-response curves for plasma and serum seemed to be parallel, but the maximal binding of the VIII:CAg from serum was lower (Figure 1).

21 patients with haemophilia A of varying severity were examined. The results are given in Table 2. All cases that had earlier been classified as severe because of VIII:C activity below 0.01 U/ml, had VIII:CAg below the level of detection, i.e. 0.01 U/ml. 2 patients, not included in Table 2, had received a factor VIII concentrate 1–6 d before the sampling. These 2 patients had a small amount of VIII:CAg in the plasma.

4 patients had moderately severe haemophilia A (VIII:C between 0.02–0.04 U/ml). The VIII:CAg in 3 of them was between 0.04–0.06 U/ml. However, 2 of these pa-

TABLE 2
Factor VIII related activities in haemophilia A of varying severity

Pat.	Fam. no.	VIII:C (U/ml)	VIII:CAg (U/ml)	VIII:Ag (U/ml)	Remarks
Severe					
1	22	<0.01	<0.01	0.86	Anticoagulant
2	32	<0.01	<0.01	1.10	
3	36	<0.01	<0.01	2.10	
4	42	<0.01	<0.01	2.13	
5	75	<0.01	<0.01	1.72	
6	76	<0.01	<0.01	0.92	
7	80	<0.01	<0.01	2.16	
8	105	<0.01	<0.01	1.12	
9	106	<0.01	<0.01	1.10	
10	121	<0.01	<0.01	1.76	
11	158	<0.01	<0.01	1.08	
12	181	<0.01	<0.01	1.14	
13	244	<0.01	<0.01	2.00	
Moderate					
14	27	0.02	0.04	1.02	(NP dose-response) (abnormally low maximal binding)
15	27	0.03	0.05	1.12	
16	156	0.04	0.06		
17	352	0.02	<0.01	1.45	
Mild					
18	100	0.05	0.03	1.58	
19	115	0.06	<0.01	1.18	
20	280	0.06	<0.01	1.14	
21	612	0.05	0.03	1.20	

TABLE 3

Factor VIII related activities in von Willebrand's disease, severe, type 1

	VIII:C (U/ml)	VIII:CAg (U/ml)	VIII:Ag (IRMA) (U/ml)
22	0.04	0.03	<0.0001
23	0.03	0.03	<0.0001

tients (nos 14 and 15), belonging to the same family, had dose-response curves that were not quite parallel to that obtained with normal plasma. The figures for these 2 patients given in Table 2, are therefore not exact but only represent the mean of the values calculated from the different dilutions. The dose-response curve for no 15 also showed an abnormally low maximal binding of antigen (Figure 1). 1 patient with VIII:C of 0.02 U/ml did not have any detectable VIII:CAg.

4 patients had mild haemophilia A (VIII:C 0.05–0.06 U/ml). 2 of them (nos 19 and 20) did not have any detectable VIII:CAg with our method. 2 patients (nos 18 and 21) had an VIII:CAg roughly equal to VIII:C.

Also 2 patients with severe von Willebrand's disease were tested. The values are

given in Table 3. VIII:C and VIII:CAg were equal in those 2 patients, neither of whom had detectable VIII:Ag. Patient no 23 received an infusion of a factor VIII concentrate. The results of the infusion studies are shown in Figure 2. VIII:C showed the typical retarded increase, as did VIII:CAg, though it was less striking as for VIII:C.

VIII:CAg was investigated also in 4 fetuses. Values from 0.08 to 0.32 U/ml were noted, the highest in the oldest fetus (20 weeks). VIII:Ag in the same fetuses ranged from 0.58 to 1.80 U/ml. Amniotic fluid did not contain any detectable VIII:CAg or VIII:Ag.

DISCUSSION

2 homologous antibodies to VIII:C were used in our immunoradiometric assay. Only one of the antibodies (from the first patient) proved to be suitable for labelling and isolation with the method used. This method included complex formation with a factor VIII concentrate, isolation of a high molecular weight complex by gel filtration and dissociation of the complex at an acid pH.

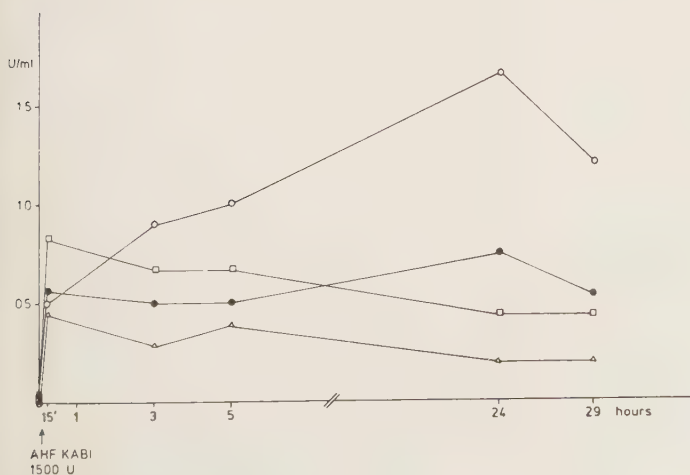


Figure 2. Effect of infusion of F VIII concentrate to a patient (23) with severe von Willebrand's disease.

○—○ VIII:C, ●—● VIII:CAg, □—□ VIII:Ag, △—△ VIII:IRCF.

The recovery of antibody activity in the final, labelled preparation was 40 %, indicating that this antibody formed stable complexes with factor VIII and that a large proportion of these complexes had a high molecular weight. In contrast, labelled antibodies from the second patient could be isolated with only a very poor yield, probably because stable high molecular weight complexes with factor VIII were formed in only small amounts (Lazarchick & Hoyer 1978).

The first step in the immunoradiometric assay consists in coating of plastic tubes with antibody-containing IgG (Peake & Bloom 1978); the second, in incubating the material to be tested in various dilutions; and the third, in detection of bound antigen with labelled antibody. This so-called sandwich technique requires that the antibodies can be bound to at least 2 different immunologic sites of the antigen molecule. When we used IgG from the first patient for coating, no dose-response curve was obtained. However, when IgG from the second was used for coating and labelled antibody from the first for detection, a typical dose-response curve was obtained for plasma dilutions from 1/4 to 1/256 with linearity between 1/16 and 1/128. A similar dose-response curve was obtained when IgG from the first or the second patient was used for coating, and labelled antibody from the second patient for detection. This strongly indicates that the antibodies from the 2 patients clearly differed in specificity. VIII:Cag seems to be monovalent for antibody of the first patient, but not for antibody of the second patient. Because of the difficulty in obtaining labelled antibody from the second patient the patients were tested with an assay consisting of IgG from the second patient for coating and labelled

antibody from the first patient for detection. The lowest level of VIII:Cag that could be confidently detected was 1 % of that in normal plasma.

With this assay there was a fairly good agreement between VIII:C and VIII:Cag of normal plasmas. The values for VIII:Cag of normal serum were consistently lower than those in plasma indicating either a smaller amount of the protein or a changed immunologic reactivity after coagulation. The latter possibility seems more probable as other workers (Peake & Bloom 1978), using a different antibody, found the same values for VIII:Cag in serum as in plasma. This discrepancy is most probably due to different specificities of the antibodies used.

All 13 severe haemophiliacs from 13 different families and with VIII:C below 0.01 U/ml had VIII:Cag, which fell below the limit of detection (< 0.01 U/ml). This indicates that severe haemophiliacs either totally lack the protein responsible for VIII:C activity or that they all have a very similar structural defect in that protein. However, Lazarchick & Hoyer (1978) found that 3 of 9 severe haemophiliacs had small amounts of VIII:Cag. Peake & Bloom (1978) tested 5 haemophiliacs with zero VIII:C. 4 of them had no detectable VIII:Cag, but 1 had a non-parallel IRMA dose-response curve indicating the presence of an VIII:Cag with altered immunologic properties. The difference may be due to differences between the antibodies used but it appears likely that severe haemophilia A may include more than one type of molecular defect.

This is even more evident in moderate and mild haemophilia A. In our 4 patients with moderately severe haemophilia we found an VIII:Cag that was approximate-

ly equal to VIII:C in 1, non-parallel dose-response curves in 2, and a not detectable VIII:CAG in 1. These data indicate the presence of abnormally reacting molecular species of VIII:CAG in some patients with moderate haemophilia A.

Various types of mild haemophilia also seem to exist. In 2 patients the VIII:CAG level equalled that of VIII:C suggesting a reduced level of a normal VIII:CAG. 2 other patients had no detectable VIII:CAG in our assay. There must therefore exist at least two different abnormalities in mild haemophilia A. Lazarchick & Hoyer (1978) found a variation in a group of mild-moderate haemophiliacs, who had a varying excess of VIII:CAG compared with that of VIII:C. As for our 3 patients (nos 17, 19 and 20), who had no detectable VIII:CAG, but 0.02–0.06 U/ml VIII:C, one must assume that they have abnormal molecules, which happen not to react with our antibodies. Different antibodies may be more or less suitable for detecting a genetic sub-variation among mild haemophiliacs.

In the patients tested with von Willebrand's disease VIII:CAG paralleled VIII:C. VIII:CAG, like VIII:C, showed the retarded increase after F VIII infusion, which is so typical of von Willebrand's disease. But the increase in VIII:CAG was not so marked as in VIII:C. It is not known why at present.

As expected (Holmberg et al 1974), VIII:CAG was demonstrated in plasma from 4 fetuses. As pointed out by Peake & Bloom (1978), an assay for VIII:CAG, such as the one described here, implies a potential possibility of a prenatal diagnosis of haemophilia A. Fetal blood samples obtained in vivo may be more or less contaminated with amniotic fluid and thus unsuitable for conventional clotting assays. Measurement

of VIII:CAG in the fetal blood should, on the other hand, be possible, since our studies showed that amniotic fluid is devoid of VIII:CAG.

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Inheritable Molecular Variants of Moderate and Mild Hemophilia A

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ABSTRACT. Factor VIII clotting activity (VIII:C) and factor VIII clotting antigen (VIII:CAg) were investigated in 54 patients with hemophilia A of moderate or mild severity. The patients belonged to 28 kindreds. The study showed a genetically determined molecular variation within hemophilia A of both moderate and mild forms. Each form can be classified into 3 types according to the content of demonstrable VIII:CAg. Type I has no demonstrable VIII:CAg, type IIa has VIII:CAg in an amount smaller than, or approximately equal to, that of VIII:C and type IIb has a larger amount of VIII:CAg than VIII:C. Affected members of one and the same kindred always have the same type of the disease.

Key words: factor VIII, hemophilia A, genetic variation, immunoradiometric assay.

Acta Med Scand 209: 11, 1981.

Hemophilia A is usually classified as severe, moderate or mild, according to the level of factor VIII clotting activity (VIII:C) in plasma measured by conventional clotting assays (2, 14). VIII:C is not measurable or less than 1% of normal, i.e. <0.01 U/ml, in severe, 0.01 – 0.04 U/ml in moderate, and 0.05 – 0.25 U/ml or even higher in mild hemophilia A.

The plasma factor with VIII:C activity probably forms a dissociable complex with a macromolecule called factor VIII related protein or antigen (VIIR:Ag). The latter can be quantified by electroimmunoassay or radioimmunoassay (3, 8, 22) and is normal in hemophilia A but deficient in von Willebrand's disease. Recently, immunoradiometric methods have been described for determining the antigen associated with VIII:C (VIII:C antigen, VIII:CAg) (6, 9, 16).

VIII:CAg is usually not detected in severe hemophilia A (6, 16), but published reports (6, 10,

11, 17) indicate that it may vary in moderate and mild hemophilia A.

This paper reports a study of VIII:C and VIII:CAg in 54 patients belonging to 28 families with haemophilia A of moderate and mild degree. The purpose was to ascertain whether there are genetically determined subtypes of moderate and mild hemophilia A.

PATIENTS

The study group consisted of 54 patients with moderate or mild hemophilia A. The patients belonged to 28 kindreds. Most of these have been described earlier and the type of hemophilia occurring in them has been classified as either moderate (VIII:C 0.01 – 0.04 U/ml) or mild (VIII:C 0.05 – 0.25 U/ml) (1, 18). In some patients the VIII:C levels differed slightly from those found on earlier occasions. In a few of them the present VIII:C values would have upgraded the disease from mild to moderate and vice versa. However, in these cases the original classification was based on repeated testing of VIII:C activity and was therefore maintained. Five patients had VIII:C values above 0.25 U/ml, but still within the subnormal range (0.30 – 0.58). Their clinical picture justified their classification as mild hemophilia A.

METHODS

Collection of blood. Citrated plasma, obtained and prepared as described elsewhere (13), was immediately frozen and stored at -60°C until examined.

Bleeding time was measured according to the method of Ivy (15).

Factor VIII clotting activity was determined with a one-stage recalcification assay (12).

Factor VIII related antigen was measured with a quantitative electroimmunoassay (7).

Abbreviations: VIII:C=factor VIII clotting activity, VIIR:Ag=factor VIII related antigen, VIII:CAg=factor VIII clotting antigen.

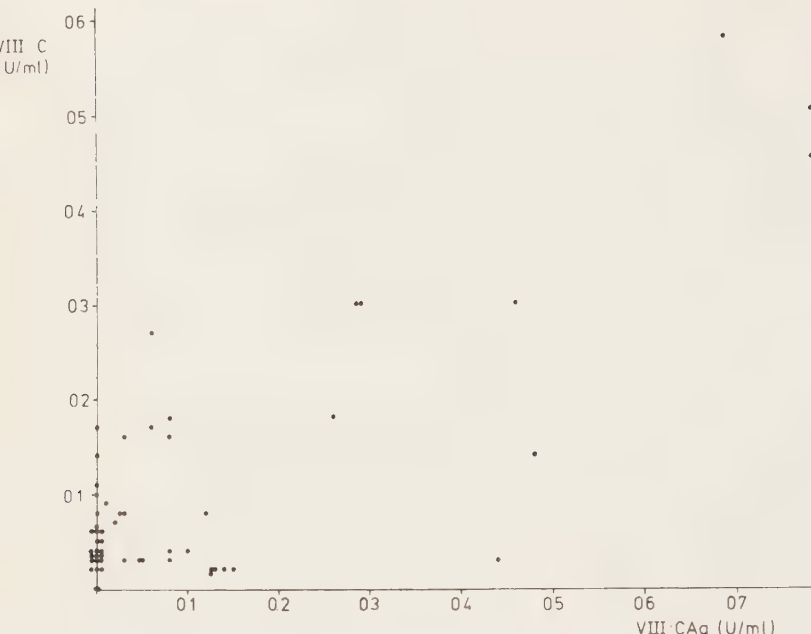


Fig. 1. VIII:C plotted against VIII:CAg in 54 patients with hemophilia A of moderate or mild severity.

Factor VIII clotting antigen was measured with an immunoradiometric assay based on 2 homologous non-hemophilic antibodies, as described in detail elsewhere (6). The method is briefly as follows: Sera from two non-hemophilic patients who have spontaneously developed high titers of inhibitor of VIII:C are used as sources of antibody. The test is performed in three steps. In the first, polystyrene tubes are coated with IgG from one of the

patients, and in the second the plasma to be tested is added in various dilutions. In the third step, purified ^{125}I labelled antibodies to VIII:C, prepared from the other patient, are added. The tubes are washed and the activity is measured in a gamma scintillator.

This two-site solid phase immunoradiometric method requires that two antigenic sites are accessible on the protein to be assayed. When normal plasma was tested, the dose-response curve for dilutions 1/16–1/128 was linear. The lowest concentration of VIII:CAg which could be measured was 0.008 U/ml, i.e. 0.8% of the concentration in pooled normal plasma. No detectable VIII:CAg is expressed as <0.01 U/ml.

Statistical methods used were *t*-test and *F*-test after metametric transformation of the variables into their common logarithms.

Table 1. VIII:C and VIII:CAg in 20 patients with moderate hemophilia A

Family no.	Pat. no.	VIII:C (U/ml)	VIII:CAg (U/ml)
I	I:1	0.02	<0.01
	II:1	0.02	<0.01
	II:2	0.03	<0.01
II	III:1	0.03	<0.01
	IV:1	0.03	<0.01
	V:1	0.04	<0.01
V	V:2	0.03	<0.01
	V:3	0.02	<0.01
	VI:1	0.03	<0.01
VI	VI:2	0.04	<0.01
	VII:1	0.03	0.03
VII	VIII:1	0.08	0.03
	VIII:2	0.03	0.05
X	IX:1	0.03	0.08
	X:1	0.03	0.44
X	X:2	0.02	0.14
	X:3	0.02	0.13
	X:4	0.02	0.13
	X:5	0.02	0.15
	X:6	0.02	0.13

RESULTS

VIII:C and VIII:CAg were measured in 54 hemophiliacs belonging to 28 kindreds. VIII:C is plotted against VIII:CAg in Fig. 1. No correlation was found in the patient series as a whole. Twenty patients (from 10 families) had moderate (VIII:C 0.01–0.04 U/ml) and 34 (from 18 families) mild hemophilia A (VIII:C 0.05–0.58 U/ml).

Table 1 gives the families with moderate hemophilia A. Two main types could easily be distinguished. The first (type I) had no demonstrable VIII:CAg, while the second (type II) had. Type II could be subdivided into one type with VIII:CAg level approximately equal to that of VIII:C (type

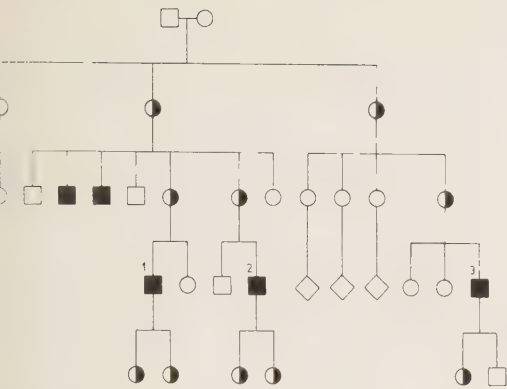


Fig. 2. Pedigree of family V with moderately severe hemophilia A, type I. None of the investigated hemophiliacs (1, 2, 3) had any detectable VIII:CAG.

la) and a second with a larger amount of VIII:CAG than of VIII:C (type IIb). Of these 20 patients, 10 (6 kindreds) had type I, 3 (2 kindreds) type IIa and 7 (2 kindreds) type IIb of moderate hemophilia A. Members of the same family always had the same type of the disease. Figs. 2 and 3 show pedigrees of family V (type I) and family X (type IIb). There was no difference in VIII:C between the three types. The difference in VIII:CAG level between types IIa and IIb was statistically significant ($p < 0.01$).

Table II shows the families with mild hemophilia A. Like in moderate hemophilia A, three types (I, IIa and IIb) could be distinguished. No detectable VIII:CAG was found in type I. In type IIa, VIII:CAG was detected, but in a smaller amount than VIII:C. In type IIb, the level of VIII:CAG was higher than that of VIII:C. Of these 34 patients, 14 (7 kindreds) had type I, 9 (5 kindreds) type IIa and 11 (6 kindreds) type IIb of mild hemophilia A. Like in moderate hemophilia A, members of the same family always had the same type of the disease. Figs. 4 and 5 show pedigrees of family XV (type I) and family XX (type IIa). One patient (XVII:2) had a transient factor VIII:C antibody after vigorous treatment with factor VIII concentrate or a burn.

There were no statistically significant differences in VIII:C between the three types of mild hemophilia A. The difference in VIII:CAG between types IIa and IIb was significant ($p < 0.01$).

Ivy bleeding time in all patients was below or only slightly above the upper normal limit (12 min).

Table II. VIII:C and VIII:CAG in 34 patients with mild hemophilia A

Family no.	Pat. no.	VIII:C (U/ml)	VIII:CAG (U/ml)
XI	XI:1	0.04	<0.01
XII	XII:1	0.05	<0.01
	XII:2	0.06	<0.01
XIII	XIII:1	0.05	<0.01
	XIII:2	0.06	<0.01
	XIII:3	0.06	<0.01
XIV	XIV:1	0.08	<0.01
XV	XV:1	0.14	<0.01
	XV:2	0.10	<0.01
	XV:3	0.06	<0.01
	XV:4	0.03	<0.01
XVI	XVI:1	0.11	<0.01
XVII	XVII:1	0.17	<0.01
	XVII:2	0.05	<0.01
XVIII	XVIII:1	0.09	0.01
XIX	XIX:1	0.16	0.03
XX	XX:1	0.07	0.02
	XX:2	0.08	0.03
	XX:3	0.27	0.06
XXI	XXI:1	0.17	0.06
	XXI:2	0.18	0.08
XXII	XXII:1	0.16	0.08
	XXII:2	0.30	0.29
XXIII	XXIII:1	0.03	0.05
	XXIII:2	0.04	0.08
	XXIII:3	0.08	0.12
XXIV	XXIV:1	0.04	0.10
XXV	XXV:1	0.18	0.26
XXVI	XXVI:1	0.30	0.46
XXVII	XXVII:1	0.14	0.48
XXVIII	XXVIII:1	0.30	0.29
	XXVIII:2	0.58	0.69
	XXVIII:3	0.45	0.78
	XXVIII:4	0.50	1.20

VIII:R:Ag was determined with electroimmunoassay in all patients. The mean value ± 1 S.D. was 1.02 ± 0.43 , 1.44 ± 0.60 and 1.31 ± 0.62 U/ml in patients with moderate hemophilia A types I, IIa and IIb, respectively. In patients with mild hemophilia the corresponding values were 1.10 ± 0.40 , 1.41 ± 0.51 and 1.36 ± 0.47 . Statistically there was no difference in VIII:R:Ag between the three types of either moderate or mild hemophilia A.

DISCUSSION

It has not yet been possible biochemically to characterise the molecular structure responsible for plasma VIII:C activity, certainly owing to its lability and low concentration. Heterologous antibodies to VIII:C have been very difficult to produce. However, antibodies to VIII:C can arise in humans

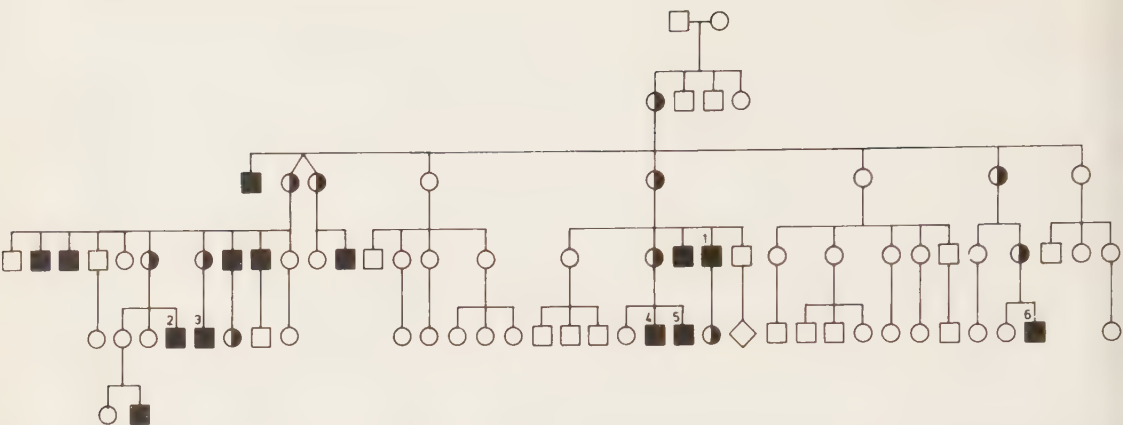


Fig. 3. Pedigree of family X with moderately severe hemophilia A, type IIb. All six investigated hemophiliacs

(1-6) had considerably higher levels of VIII:C_{Ag} than of VIII:C.

and be used in the study of its molecular properties and the biochemical defect in hemophilia A. Some early studies using neutralisation assays (4, 5, 9) have suggested the existence of two molecular types of hemophilia A. But neutralisation tests are not very discriminating. More recent and sensitive assays of VIII:Cag use radiolabelled, purified homologous antibodies. With such an assay we have earlier studied VIII:Cag in 14 patients with severe hemophilia A (6). None of them had any demonstrable VIII:Cag. This indicates that severe hemophiliacs either have no factor VIII:C protein at all or a very similar severe structural defect in that protein. However, other investigators, using similar immunoradiometric assays, have found detectable

antigen in some patients with severe hemophilia A, indicating heterogeneity (10, 16, 17, 19).

Earlier studies also suggest that moderate as well as mild hemophilia A includes more than one type of molecular defect (6, 11, 17). But it has not been shown whether such a variation is genetically determined.

In the present investigation we examined 54 patients with moderate or mild hemophilia A. Twelve kindreds were available in which more than one affected member could be examined. In both moderate and mild hemophilia A two clearly separated main types were distinguished, one without (type I) and one with (type II) demonstrable antigen. Type II can be subdivided into one group (IIa) with VIII:CAg lower than, or approximately equal to, VIII:C and one (IIb) with a higher level of demonstrable antigen than of activity. There is a homogeneity within each family, since affected

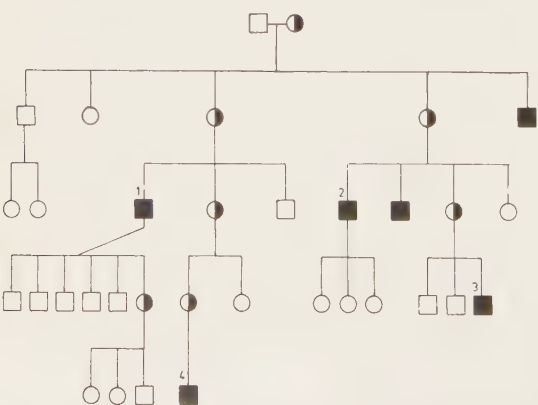


Fig. 4. Pedigree of family XV with mild hemophilia A, type I. None of the investigated hemophiliacs (1-4) had any detectable VIII:Cag.

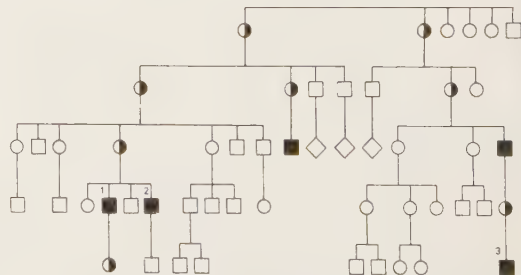


Fig. 5. Pedigree of family XX with mild hemophilia A, type IIa. All three investigated hemophiliacs (1, 2, 3) had VII CAg but in a smaller amount than VIII:C.

members of one and the same family invariably had the same type.

There may be a slight variation within a family, but the general pattern is always the same. One member (X:1) of family X (moderate IIb) had higher VIII:C/Ag than the other members of the family. He also had a higher VIIIR:Ag, 2.35 U/ml, which might be due to a reactive process (7). The higher level of VIII:C/Ag was probably secondary to the raised level of VIIIR:Ag. Despite the rise in VIII:C/Ag, VIII:C activity did not rise appreciably.

The data presented here clearly suggest that the molecular variants demonstrated are inheritable. At least three, but probably more, genetically determined variants of moderate as well as of mild hemophilia A seem to exist.

The immunoradiometric assay used requires the presence of two antigenic sites on the factor VIII:C molecule for reaction with the two antibodies used. A defective factor VIII:C molecule with only one immunoreactive site will not be detected by the assay. In type I one must assume that one or more antigenic sites are missing in the molecule or that they are not accessible to the antibodies used. In spite of such a defect, the VIII:C protein, whether present in normal or decreased amounts, retains some biological activity. In type II one must assume that the VIII:C molecule is altered in such a way that its antigenic properties are better preserved but that its biological activity is not higher than in type I.

The clinical severity of the disease was assessed in all of the 54 patients. It appears that the levels of VIII:C/Ag do not vary with the clinical severity of the disease.

Development of an anticoagulant is a serious complication in the treatment of hemophilia and affects patients with the severe form of the disease and very seldom those with moderate or mild forms (8). One of our patients with mild hemophilia A (type I, i.e. the type without detectable antigen, developed a transient factor VIII:C antibody after rigorous treatment. Patients with such a type of hemophilia A might be more prone to develop an anticoagulant.

Immunoradiometric assays of VIII:C/Ag have been used successfully for prenatal detection of severe hemophilia A in fetuses at risk. Hemophilia, classified as moderate according to the laboratory tests, may sometimes be clinically indistinguishable from hemophilia A of severe form. Prenatal diagnosis might therefore be indicated also in fetuses at

risk for this form. If an immunoradiometric assay were to be used as the only diagnostic test, prenatal diagnosis can obviously be offered only to carriers from families in which affected members are lacking in antigen (type I).

Our conclusion is that there are several inheritable molecular variants in both moderate and mild hemophilia A. There are probably more genetic variants than we have been able to demonstrate. Further knowledge of the genetic variation of hemophilia A might, perhaps, be acquired if the patients were tested with different homologous as well as heterologous (21) antisera. Such studies would perhaps reveal an even greater complexity and possibly even subgroups of severe hemophilia A.

ACKNOWLEDGEMENTS

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F VIII:CAg IN HAEMOPHILIA A
A COMPARISON BETWEEN IRMA:s USING HAEMOPHILIC AND SPONTANEOUS ANTIBODIES

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ABSTRACT

Factor VIII clotting antigen (VIII:CAg) was measured with two different immunoradiometric assays, one utilizing two acquired antibodies and one a haemophilic antibody, in order to investigate the genetic heterogeneity in haemophilia A of different severity and to what extent VIII:CAg levels depends on the antibody used. Fifteen severe haemophiliacs were, with one exception, classified as CRM-. Eleven moderate and 21 mild haemophiliacs were investigated and there was a good agreement between the results obtained with the different antibodies. The study confirms the division of both moderate and mild haemophilia A into 3 subgroups, proposed in an earlier investigation. It also favours the opinion that in haemophilia A there is a quantitative reduction rather than a qualitative defect in the protein responsible for factor VIII clotting activity (VIII:C).

INTRODUCTION

Haemophilia A is usually classified as severe, moderate or mild according to the level of factor VIII clotting activity (VIII:C) in plasma measured by conventional clotting assays (1, 2). The development of sensitive immunoradiometric assays (3-7) has allowed quantification of the antigen in plasma associated with VIII:C, factor VIII clotting antigen (VIII:CAg). Studies with such methods have demonstrated a genetical heterogeneity of haemophilia A (3-8). All the immunoassays used, whether one- or two-site techniques, rely on human, spontaneous or haemophilic, antibodies against VIII:C. Conflicting results of the VIII:CAg levels in haemophilia A of different severity have

been obtained. It is thus still unclear whether in haemophilia A, VIII:CAg has a normal or abnormal structure or whether it is present in normal or decreased amounts or totally lacking. Part of the heterogeneity shown to exist in haemophilia A might be due to variable specificity of the antibodies used and to different assay techniques. This paper reports VIII:CAg measured in haemophiliacs with two different methods; one utilizing two non-haemophilic antibodies and one a haemophilic antibody, but employing the same basic immunoradiometric assay technique. The purpose is to investigate whether the results of VIII:CAg determination depends on the antibody used.

MATERIAL AND METHODS

Patients. The study group consisted of 47 patients with haemophilia A. The disease was severe in 15 of the patients (VIII:C <1 U/dl), moderately severe in 11 (VIII:C 1-4 U/dl) and mild (VIII:C 5-25 /50/ U/dl) in 21. 1 U = the factor VIII activity, VIII:C, in 1 ml of pooled normal human plasma.

Collection of blood. Citrated plasma, obtained and prepared as described elsewhere (9), was immediately frozen and stored at -60°C until examined.

Factor VIII clotting activity (VIII:C) was determined in a one-stage recalcification system with plasma from a patient with severe haemophilia A as test base (10).

Factor VIII clotting antigen (VIII:CAg) was determined with a two-site immunoradiometric assay (IRMA) using both haemophilic and non-haemophilic human antibodies.

The IRMA using non-haemophilic antibodies was carried out as described in detail elsewhere (5). In this method two spontaneously arisen antibodies are used. The titres of the immune-sera were 250 U/ml (approx. 500 Bethesda U/ml) and 500 U/ml (approx. 1000 Bethesda U/ml), respectively. The first one was used for coating the tubes and the second one for labelling and use in the third step in the assay. The lowest concentration of VIII:CAg that can be measured is 0.8 U/dl. No detectable VIII:CAg is here expressed as <1 U/dl.

The source of the haemophilic antibody was plasma from a 60 year old man with severe haemophilia A. He had had an anticoagulant against VIII:C in low titre for many years. After substitution therapy before surgery of a pseudo-tumour, the antibody titre rose to 1100 U/ml (approx. equal to 2200 Bethesda U/ml). The preparation of labelled antibody and the immunoradiometric technique were identical with the earlier published assay (5) with the exception that the same antibody is used also for coating. The lowest concentration that can be measured is 0.5 U/dl. In plasma from 22 normal individuals the mean value $\pm$ 2 SD was 115 ± 52 for VIII:C and 92 ± 56 for VIII:CAg. The intra- and interassay coefficients of variation were less than 10 %.

RESULTS

Levels of VIII:CAg measured with a haemophilic antibody were compared to those measured with non-haemophilic antibodies in 44 patients with haemophilia A.

Table I shows the results in 15 patients, from 14 families, with severe haemophilia A. None of the patients, except 14:1, who had a very small amount measured with the spontaneous antibodies, 1.6 U/dl, had demonstrable antigen with any method.

TABLE I

F VIII:C and F VIII:CAg Measured with Haemophilic (HA) and Acquired (AA) Antibodies in 15 Patients with Severe Haemophilia A

Fam. no./Pat. no.	VIII:C (U/dl)	VIII:CAg HA (U/dl)	VIII:CAg AA (U/dl)
1:1 - 12:1*	<1	<1	<1
13:1	<1	<1	<1
13:2	<1	<1	<1
14:1	<1	<1	1.6

* 1:1 - 1:5 had an anticoagulant against VIII:C

TABLE II

F VIII:C and F VIII:CAg Measured with Haemophilic (HA) and Acquired (AA) Antibodies in 11 Patients with Moderate Haemophilia A

Fam. no./Pat. no.	VIII:C (U/dl)	VIII:CAg HA (U/dl)	VIII:CAg AA (U/dl)
1:1	1	<1	<1
2:1	2	<1	<1
2:2	3	<1	<1
3:1	1	<1	<1
3:2	2	<1	<1
3:3	3	<1	<1
3:4	4	<1	<1
4:1	3	1	1
4:2	4	1	2
5:1	2	13	15
5:2	2	14	15

TABLE III

F VIII:C and F VIII:Cag Measured with Haemophilic (HA) and Acquired (AA) Antibodies in 21 Patients with Mild Haemophilia A

Fam. no./Pat. no	VIII:C (U/dl)	VIII:Cag HA (U/dl)	VIII:Cag AA (U/dl)
1:1	5	<1	<1
2:1	5	<1	<1
3:1	11	<1	<1
4:1	5	<1	1.4
4:2	6	<1	<1
4:3	10	<1	<1
4:4	14	<1	<1
5:1	4	<1	1.2
6:1	3	3	4
7:1	16	3	1
8:1	7	2	2
8:2	8	2	4
8:3	27	6	5
9:1	17	6	4
9:2	18	8	5
10:1	9	1	4
11:1	16	3	1
11:2	16	8	13
12:1	16	26	23
13:1	35	46	49
14:1	14	44	60

Table II shows 11 patients from 5 families with moderate haemophilia A. Seven of the patients from 3 families had no detectable antigen with either method. Two patients from 1 family had VIII:Cag approximately equal to VIII:C with both antibodies and two, from 1 family, had antigen exceeding the activity but still in the subnormal range. There was a good agreement between VIII:Cag levels measured with the two methods in all the patients with moderate haemophilia A.

Table III shows 21 patients (from 14 families) with mild haemophilia A. Eight patients had no demonstrable antigen, 12 had antigen levels approximately equal to or less than VIII:C and one patient had antigen in excess of activity with either of the methods used. In two patients, 4:1 and 5:1,

there was a difference between the methods, since small amounts of VIII:CAg were detected with the spontaneous antibodies but not with the haemophilic antibody. In the other 3 members of family 4, however, the two methods gave the same results.

DISCUSSION

In severe haemophilia A most investigators (4, 5, 7) have found no patient or only an occasional one to have detectable amounts of VIII:CAg. No patients with severe haemophilia A with normal amounts of VIII:CAg have been discovered. In the present study only one of 15 severe haemophiliacs had a small amount of VIII:CAg, detectable only with the spontaneous antibodies. This finding favours the view that in severe haemophilia A almost all patients lack the protein associated with VIII:C rather than having a structural defect that changes its antigenic properties so that it is not recognized by antibodies. Other investigators have found a slightly higher proportion of CRM+ variants in severe haemophilia A (3, 6), although the majority were CRM-. The difference might be due to the assay-technique. We, like some other investigators who have found very few CRM+ variants (4, 7), use a two-site solid phase technique that requires the presence of at least two antigenic sites on the molecule. One-site liquid phase techniques (3, 6) seem to detect a higher proportion CRM+ cases. In the CRM+ variants there seems to be a quantitative reduction in the protein since there is a good agreement between results obtained by different antibodies (6).

A genetically determined heterogeneity has also been shown to exist in moderate and mild haemophilia A (8). The results of the present study justifies the subdivision of the cases, made in an earlier investigation (8). Type I has no detectable VIII:CAg, type IIa has VIII:CAg levels approximately equal to or less than VIII:C and type IIb has antigen exceeding activity but still subnormal.

In the 11 moderate haemophiliacs investigated, there was a good agreement between the results obtained by the haemophilic and the acquired antibodies. In the 21 patients with mild haemophilia A there was also a good correspondence between the two methods using different antibodies, except in two cases, 4:1 and 5:1 who had small amounts of antigen detectable only with the spontaneous antibodies. The results of VIII:CAg determinations thus seem to be fairly independent of antibody specificity. The conclusion is that in mild and moderate haemophilia A, the VIII:C protein is present in reduced amounts, with the biological activity preserved in type IIa, but reduced in type IIb. In type I, lacking detectable antigen, one must assume that a major immuno-reactive site in VIII:C is missing since the molecule is not recognized by any of the methods used. The antigenic site must be apart from the active site of the protein, since the molecule retains some biological activity.

The recognition of a genetic heterogeneity in haemophilia A has become important during the last few years when prenatal diagnosis of haemophilia in fetuses at risk of the disease has been introduced. Immunoradiometric assays are very useful in CRM- kindreds and in kindreds where affected members have very small amounts of antigen. Although the present and other investigations show a good agreement between VIII:CAg levels determined by different antibodies, it might be possible to find other antibodies which can distinguish between normal VIII:CAg and the VIII:CAg of CRM+ haemophilia, and make prenatal diagnosis feasible also in those rare types.

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GENETIC VARIANTS OF HAEMOPHILIA B DETECTED BY IMMUNOPADIOMETRYC ASSAY

IMPLICATIONS FOR PRENATAL DIAGNOSIS

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SUMMARY

50 patients with haemophilia B belonging to 29 kindreds were investigated with a highly sensitive immunoradiometric assay based on a homologous antibody to f. IX. The assay measures factor IX antigen (f.IX:Ag) in plasma down to 0.025 U/dl. Seventeen of 18 investigated patients with severe haemophilia B had very little or no f.IX:Ag. Also 4 of 9 patients with moderately severe disease had very low antigen levels, approximately equal to their factor IX clotting activity (f.IX:C), whereas the other 5 had antigen in excess of activity. Of the 23 investigated patients with mild haemophilia B, 20 had f.IX:Ag approximately equal to f.IX:C, whereas 3 had normal amounts of antigen. One family with mild disease was found to have a possible variant of haemophilia B Leyden, earlier described in a few families with moderately severe disease. No haemophilia B_M variants, characterized by prolonged prothrombin time with bovine brain thromboplastin, were found. We have earlier shown that the immunoradiometric assay of f.IX was useful in the prenatal evaluation of one fetus at risk for haemophilia B. The present study shows that the assay can be applied for prenatal diagnostic purposes in the vast majority of carriers of severe haemophilia B and in about half of the carriers of moderately severe disease.

INTRODUCTION

Haemophilia B is usually classified as severe, moderate or mild, according to the level of factor IX clotting activity, f.IX:C. The existence of molecular variants of haemophilia B was detected after it had become possible to determine factor IX antigen, f.IX:Ag, using different assays (1, 7, 9, 11, 16, 17, 19, 20, 21, 22). CRM⁻ (cross reacting material negative) variants have no demonstrable f.IX:Ag; CRM⁺ variants have normal amounts, and CRM_R variants amounts reduced more or less proportional to f.IX:C. B_M variants are characterized by prolonged prothrombin time with bovine brain thromboplastin (6, 8). Most of the immunological f.IX methods described are not very sensitive and they all depend upon the specificity of the antibody used. Only one method seems to have been capable of detecting f.IX:Ag levels below 2% of normal (20). Detailed reports of cases with antigen content below 10% of normal are still scanty.

A very sensitive immunoradiometric assay using a homologous antibody has recently been described and used in the prenatal diagnosis of one case of haemophilia B (4). In the present study 50 haemophilia B patients were investigated with this immunoradiometric assay in order to characterize genetic variants including those with very low levels of f.IX:Ag. The practical purpose of the study was to ascertain to what extent immunological methods are applicable in prenatal diagnosis of haemophilia B.

MATERIAL AND METHODS

Patients. The study group consisted of 50 patients with haemophilia B belong to 29 kindreds. All the patients were informed about the purpose of the investigation and gave their consent. The disease was severe in 18 of the patients (f.IX:C < 1 U/dl), moderately severe in 9, (f.IX:C 1-4 U/dl) and mild (f.IX 5-25 U/dl) in 23. (1 U = the factor IX activity, f.IX:C, in 1 ml of pooled normal human plasma.)

Collection of blood. Citrated plasma, obtained and prepared as described elsewhere (12), was frozen immediately and stored at -60° until examined.

Factor IX clotting activity (f.IX:C) was determined in a one-stage recalcification system with plasma from a patient with severe haemophilia B as test base (13). Normal range 60-140%.

Factor IX clotting antigen (f.IX:Ag) was determined with an immunoradiometric assay (4). Serum from a patient with haemophilia B, who had developed an inhibitor to f.IX, was used as source of antibody. The titre against f.IX was 5000 U/dl (one unit is defined as the amount that neutralizes 1 U of f.IX during incubation for 2 hours at 37°C . 1 U/dl is approximately equal to 2 Bethesda U/dl). The method is described in detail elsewhere (4).

When normal plasma was tested a linear dose response curve was obtained for dilutions 1/128-1/2048. The mean and standard deviation in 24 normals were 108 ± 27 U/dl. f.IX:C in the same samples was 95 ± 24 U/dl. The coefficient of correlation between antigen and activity was 0.74 ($p < 0.001$). The lowest concentration of f.IX:Ag that can be measured is 0.025 U/dl, i.e. 0.025%. No detectable f.IX:Ag is here expressed as < 0.1 U/dl. At low levels (1:100, 1:1000, 1:2000) of normal pooled plasma, the inter- and intra-assay coefficients of variation are always less than 10%.

P & P was measured according to the method of Owren (15), which measures the sum of the vitamin K-dependent coagulation factors, i.e. prothrombin, factor VII and factor X. Normal range 80-120%.

Thrombotest, which is a variant of the P & P method using ox-brain thromboplastin, was performed by thrombotest reagent from Nyegaard, Oslo, Norway. Normal range 80-120%.

One-stage prothrombin time was determined with the method of Owren using thromboplastin prepared from human and bovine brains (14).

RESULTS

Table I gives the families with severe haemophilia B. Thirteen of the patients had no demonstrable antigen and 4 had a very small amount (below 1 U/dl). Patients 3:1 and 4:1 had had an anticoagulant against f.IX for many years. Of the 18 patients with the severe type of the disease, only one had antigen in excess of activity, but still in the subnormal range (26 U/dl).

Table II shows the families with moderate haemophilia B. Two groups could be distinguished. In the first f.IX:Ag was approximately equal to f.IX:C (4 patients from 3 kindreds), but in the second antigen was much higher than activity, but still in the subnormal range (5 patients from 3 kindreds). Members of the same family always had the same pattern and there was no difference in clinical severity between the two groups. One patient, 17:1, was a heterozygous woman with an unusually low level of f.IX (2).

Table III shows the families with mild haemophilia B. Two groups could be distinguished. In the first f.IX:Ag was approximately equal to f.IX:C (18 patients from 5 kindreds) and in the second f.IX antigen exceeded the activity and was in the normal range (3 patients from 3 kindreds). Members of the same family always had the same pattern except in family 27. Fig. 1 shows the largest family with 8 affected members, all of whom had subnormal and very similar f.IX:Ag levels.

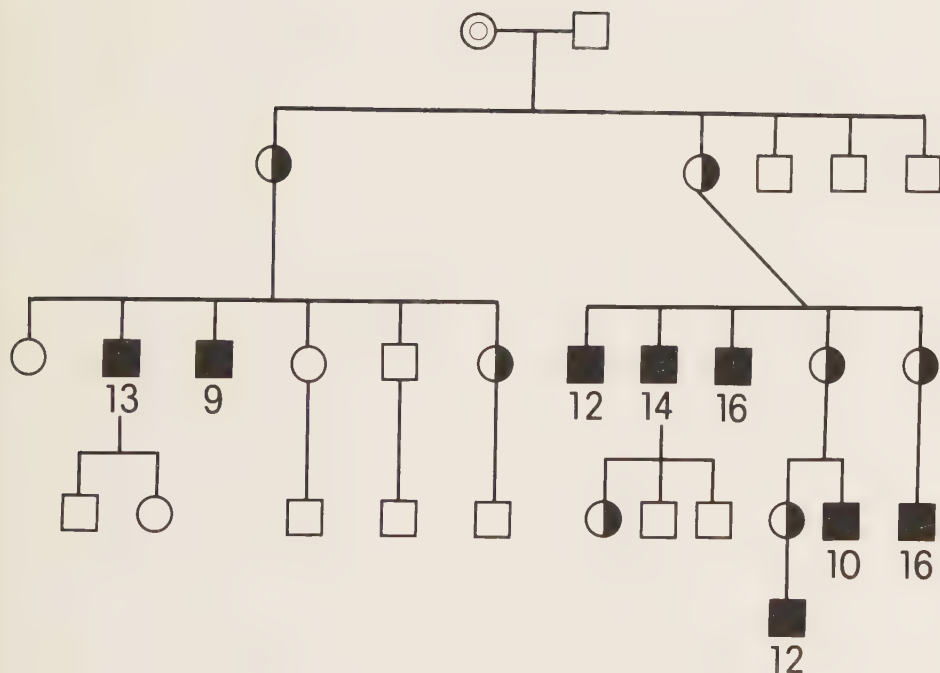


Fig. 1 Pedigree of family 22 with mild haemophilia B. The 8 affected members had very similar f.IX:Ag levels.

The carriers status was confirmed by low f.IX:C levels.

⊙ indicates a probable carrier.

In family 27, two twin brothers born in 1953 were found to have mild haemophilia B in 1960. Their f.IX:C values at that time were 18 U/dl and 25 U/dl, respectively. Mild haemophilia B was diagnosed in their two year older brother, 27:1 in 1956, and his f.IX:C at that time was 12 U/dl. When the family was reexamined in the present investigation we found repeatedly normal values in 27:2 and 27:3 and 4 U/dl in 27:1.

All investigated patients had normal P & P and one-stage prothrombin time tested with human brain thromboplastin.

Thrombotest and one-stage prothrombin time with bovine brain thromboplastin were performed in all the patients in order to find out whether any had the B_M variant. The values for both thrombotest and prothrombin time proved normal in all the patients.

DISCUSSION

We investigated 50 patients with haemophilia B with a new immunoradiometric assay based on a human antibody. Our assay can detect f.IX:Ag down to a concentration of at least 0.025% of that in normal plasma and has a high degree of accuracy and reproducibility also at very low levels. It is therefore suitable also for examining patients with low levels of f.IX:Ag and capable of revealing a possible heterogeneity in this group.

Of the 18 patients examined with severe haemophilia B, 13 had no detectable f.IX:Ag, 4 had tiny amounts (below 1 U/dl) and one a substantial amount (26 U/dl). Thus a fairly uniform pattern was found in severe haemophilia B. The results obtained with our immunological method and with the activity assay (sensitivity limit 1 U/dl) were concordant in all cases but one.

Our results differ somewhat from those reported by other workers. Among 10 severe haemophiliacs investigated by Thompson (1977), only 2 had f.IX:Ag below 2 U/dl. Nine severe haemophiliacs investigated by Yang (1978) had low and 3 had normal levels of f.IX:Ag. Only 9 of 35 patients with severe and moderate haemophilia B investigated by Ørstavik (1979) had f.IX:Ag below the concentration demonstrable by the method (2 U/dl). In the other studies on record (1, 7, 9, 16, 21) it was not possible to detect f.IX:Ag levels below 7-10 U/dl).

The difference between our results and those of Thompson, Ørstavik and Yang might be explained by differences in specifi-

city of the antiserum used. We use a homologous antibody which can be expected to have a narrow specificity. Heterologous antibodies used by most other workers probably have a broader specificity and thus a higher capacity to detect abnormal molecular variants since they may be directed against a greater number of immuno-reactive sites on the factor IX molecule than homologous antibodies.

About half of the patients with moderate haemophilia B had a very low level of f.IX:Ag, i.e. largely equal to their f.IX:C. When severe and moderate haemophilia B were taken together 21 patients from 17 kindreds had at most minute amounts of f.IX:Ag and could therefore be regarded as CRM⁻ for practical purposes. In contrast, 6 patients from 4 kindreds had substantial amounts and could be classified as CRM_R⁺.

Twentythree patients with mild haemophilia B were investigated. Twenty of them were CRM_R variants, mostly with f.IX:Ag just as low or even lower than f.IX:C. The remaining 3 were CRM⁺ variants. These 3 presumably have a f.IX molecule that is structurally altered in such a way that its antigenic properties are better preserved but its biological activity is not higher than in other patients with mild haemophilia B.

In families 22 and 25 several members were investigated. The range of variation of f.IX:Ag values was narrower than that of f.IX:C values (F-test: variance ratio 19.9, $P < 0.001$). This was certainly because antigen can be measured with greater accuracy.

In family 27 the three members were of approximately the same age and mild haemophilia B had been diagnosed 20 years ago from their f.IX:C values (12, 18 and 25 U/dl) measured with the same method as today. In the present study they were investigated several times and we always found normal f.IX:C and f.IX:Ag values in 27:2 and 27:3, but a low f.IX:C value in spite of a normal f.IX:Ag in 27:1. This family might have a

haemophilia B Leyden variant, which has been described in a few families with moderate haemophilia B and is characterized by a diminishing bleeding tendency and increasing f.IX:C with increasing age (18). If so, it would mean that a molecular defect had disappeared with time in 2 of the patients.

Haemophilia B_M is characterized by prolonged prothrombin time with bovine thromboplastin (6, 8). No B_M variants were detected in the present study. Neither have such variants been found in some earlier studies (17). This is probably because the mutant gene responsible for the B_M variant is not widely spread in the world.

Genetic counselling of pregnant carriers of haemophilia, now includes not only a recommendation of fetal sex determination. If the fetus is a male the woman is offered further prenatal evaluation. Prenatal diagnosis of haemophilia cannot, in our hands, be based upon a clotting assay of fetal blood samples obtained at fetoscopy (10), since such samples are often mixed with amniotic fluid, which disturbs clotting analysis. We have therefore resorted to immunological methods when examining for haemophilia B, as well as haemophilia A, before birth (3, 5). Our immunoradiometric method for f.IX:Ag based upon a human antibody has proved useful for that purpose since it is sensitive enough to detect the low levels of f.IX:Ag present in normal 16-20 week fetuses (4). Prenatal evaluation of a fetus can only be offered carriers in families with CRM⁻ variants of the disease and should not be attempted in an individual case before the genetic variant in the family has been determined by examination of an affected member. In conclusion, it should be possible to use the immunoradiometric assay for prenatal diagnostic purposes in 80% of families with severe and moderate haemophilia B. In mild haemophilia B prenatal evaluation of the fetus is not indicated.

Table I. F.IX:C and f.IX:Ag in 18 patients with severe haemophilia B

Family: Patient no.	IX:C (U/dl)	IX:Ag (U/dl)
	Normal range 60-140%	Normal range 54-162%
1:1	< 1	< 0.1
1:2	< 1	< 0.1
2:1	< 1	< 0.1
2:2	< 1	< 0.1
2:3	3	0.9 [*]
3:1	< 1	< 0.1 ^{**}
4:1	< 1	< 0.1 ^{**}
5:1	< 1	< 0.1
6:1	< 1	< 0.1
7:1	< 1	< 0.1
8:1	< 1	< 0.1
9:1	< 1	< 0.1
10:1	< 1	< 0.1
11:1	< 1	0.2
12:1	< 1	0.5
13:1	< 1	0.5
14:1	< 1	0.6
15:1	< 1	26.0

^{*} 2:3 had received f.IX concentrate 5 days prior to examination

^{**} 3:1 and 4:1 had developed an anticoagulant against factor IX

Table II. F.IX:C and f.IX:Ag in 9 patients with moderate haemophilia B

Family: Patient no.	IX:C (U/dl)	IX:Ag (U/dl)
	Normal range 60-140%	Normal range 54-162%
16:1	1	1.5
16:2	3	4.0
17:1	4	1.7*
18:1	3	2.0
19:1	1	26.0
19:2	1	30.0
20:1	2	35.0
20:2	2	36.0
21:1	3	44.0

* 17:1 is a heterozygous woman with an unusually low level of f.IX:C

Table III. F.IX:C and f.IX:Ag in 23 patients with mild haemophilia B

Family: Patient no.	IX:C (U/dl) Normal range 60-140%	IX:Ag (U/dl) Normal range 54-162%
22:1	10	9
22:2	16	10
22:3	15	12
22:4	15	12
22:5	28	13
22:6	36	14
22:7	12	16
22:8	39	16
23:1	16	9
23:2	28	13
23:3	26	18
24:1	22	12
25:1	15	13
25:2	21	14
25:3	21	14
25:4	18	18
25:5	27	18
26:1	37	41
27:1	4	79
27:2	89*	96
27:3	96*	100
28:1	7	110
29:1	6	115

* 27:2 and 27:3 have been diagnosed as mild haemophiliacs in earlier investigations

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HAEMOPHILIA A AND B - TWO YEARS EXPERIENCES OF
GENETIC COUNSELLING AND PRENATAL DIAGNOSIS

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ABSTRACT

Haemophilia A. Thirty-one pregnant women, obligate or probable carriers of haemophilia A, requested prenatal diagnosis if sex determination showed the foetus to be a male. In 11 of the 31 cases the foetuses were females; in 2, the genetic variant of the disease rendered prenatal diagnosis impossible; and in 2, the mother aborted spontaneously. From the remaining 16 male foetuses blood samples were obtained in utero in the 17th to 20th week of gestation. Examination of the samples showed that 11 of the foetuses were unaffected and 5 affected.

Haemophilia B. Three carriers of haemophilia B had male foetuses. Examination of foetal blood obtained in utero showed that these three foetuses were affected.

Confirmation. All women with an affected foetus requested termination of pregnancy. In one of the cases of abortion, no blood was obtained for confirmative examination. In the remaining cases, the prenatal prediction was confirmed in the abortus or in the child after birth; three women are still pregnant.

INTRODUCTION

Haemophilia A and B are sex-linked inheritable diseases affecting half of the male offspring of female carriers. Diagnosis of the conditions and their classifications as severe, moderate or mild is based on the coagulation activity of factor VIII (VIII:C) and factor IX (IX:C), respectively. The availability of substitution therapy and regular prophylaxis, especially of infants and children with respectively factor VIII and factor IX concentrates has substantially improved the prognosis of severe haemophilia (1). Nevertheless, haemophilia is still a serious disease, trying for both the patient and his family. In about 10% of all cases of the disorder the patient, most often while still very young, develops antibodies, so-called anticoagulants, to the substitution preparation given. This is a serious complication limiting the possibilities of controlling haemorrhages.

Many female members of haemophilic families have refrained from having children. But now that technical advances in amniocentesis have made prenatal determination of the sex of the foetus possible, some women prefer simply to have all male foetuses aborted. Such a choice implies a 50% risk of abortion of a healthy male foetus.

Recent improvement of foetoscopy and the ultrasound technique have made it possible to sample foetal blood in the 18th-20th week of gestation without any admixture of maternal blood (2-5). Contamination with amniotic fluid can often not be avoided. Even slight contamination of the foetal samples with amniotic fluid, with its content of thromboplastic substances, precludes determination of VIII:C and IX:C.

The elaboration of sensitive immunoradiometric methods for determining factor VIII and factor IX coagulation antigen (VIII:CAG, IX:Ag), and determination of the normal range of these values in foetuses, have enabled prenatal diagnosis of haemophilia A as well as of haemophilia B (6-9).

This paper reports two years of experience with genetic counselling and prenatal diagnosis of haemophilia A and B.

CLINICAL MATERIAL

Haemophilia A. The material consisted of 31 pregnant women who were genetically obligate carriers or were at 50% risk of being carriers, together with the results of laboratory studies i.e. low level of VIII:C. All of them had received genetic counsel and been informed about the foetal blood sampling technique and its risks. All requested determination of the sex of the foetus and if it was a male, also examination of it for haemophilia and interruption of pregnancy if the foetus was affected. They were, however, informed that they could change their minds during the diagnostic procedures.

One of the 31 women aborted spontaneously before the sex of the foetus could be determined. Two women belonged to families where the genetic variant of the disease rendered prenatal diagnosis impossible. The sex of the foetuses was determined by chromosome analyses of the amniotic fluid in the 15th-16th gestational week in the remaining 28 women. Eleven of the foetuses were females and in these cases the pregnancy was continued. In the remaining 17 cases efforts were made to obtain foetal blood in the 18th-20th gestational week. In one case foetoscopic blood sampling failed owing to bleeding into the amniotic cavity. The woman chose to continue the pregnancy but aborted spontaneously a couple of weeks later. In the remaining 16 women a prenatal diagnosis was established by examination of foetal blood.

Haemophilia B. The material consisted of 3 female carriers, including one with an extremely low content of IX:C, about 3%. All the foetuses were males and a prenatal diagnosis in the 18th-20th week of gestation was established by examination of foetal blood.

METHODS

Factor VIII coagulant activity (VIII:C) was determined with a biological method in a recalcification system (10). Normal range 60-160%. Severe haemophilia A <1%, moderate 1-4% and mild 5-25%.

Factor VIII coagulant antigen (VIII:CAg) was determined with an immunoradiometric assay based on human antisera against VIII:C, from a multitransfused haemophiliac (11). Normal range 40-150 U/dl. Sensitivity down to 0.5 U/dl.

von Willebrand factor, factor VIII related antigen (VIII:RAg) was determined with an immunoradiometric assay based on a rabbit antibody (12). Normal range 40-175 U/dl. Sensitivity down to 0.05 U/dl.

Factor IX coagulant activity (IX:C) was determined with a biological method in a recalcification system (10). Normal range 60-140%. Severe haemophilia B <1%, moderate 1-4% and mild 5-25%.

Factor IX coagulant antigen (IX:Ag) was determined with an immunoradiometric assay based on a human antibody from a patient with haemophilia B (9). Normal range 60-160 U/dl. Sensitivity down to 0.025 U/dl.

In most cases foetal blood was obtained by puncture of a placental vessel at foetoscopy. In 7 of 10 of the Danish cases, blood was obtained by heart puncture under the guidance of ultrasound. The method was first tried in a case where foetoscopy had failed. The trial proved succesful and provided a possibility of obtaining blood specimens of absolutely uncontaminated foetal blood.

The samples were obtained as follows. By ultrasound scanning the duration of pregnancy was estimated from BPD (biparietal diameter). Scanning was then done with a special puncture guide (linear Aloka equipment), which enables insertion of the cannula (15 cm long and with an outer diameter of 1.2 mm), directly to the foetus. During insertion the tip of the cannula is visualised

on the oscilloscope. The cannula is advanced until its' tip reaches the thorax of the foetus. A second cannula (18 cm long and with an outer diameter of 0.7 mm) is then inserted through the first one. Under oscilloscopic control the cannula is inserted into the ventricle and 0.3-0.5 ml of pure foetal blood is withdrawn.

Kleihauer-Betkes staining technique and determination of foetal haemoglobin were used for deciding whether the blood samples obtained at foetoscopy were of foetal origin. Sometimes a Coulter Counter-Channelyzer was used, which measures the erythrocyte volume (erythrocytes in a foetus are 50% larger than in the mother).

Any admixture of amniotic fluid could be calculated from the EVF of the foetal sample. EVF is 37% in undiluted fetal blood in the 20th week of gestation (13). In some cases the number of red blood cells in the foetoscopic sample was used instead, which is approximately 2.8×10^6 in the 20th week of gestation.

RESULTS

Table I gives the previously established reference values for VIII:CAg, VIIIR:Ag, the ratio VIII:CAg/VIIIR:Ag and IX:Ag for normal fetuses in the 16th-20th week of gestation. Small amounts of IX:Ag could be demonstrated in the amniotic fluid, but no VIII:CAg or VIIIR:Ag.

Haemophilia A. Undiluted or practically undiluted samples of foetal blood were obtained from 11 of the 16 fetuses. In the remaining 5 cases the samples were contaminated but could be used after correction for the dilution.

Eleven fetuses had normal values for VIII:CAg and a normal VIII:CAg/VIIIR:Ag ratio and were judged as unaffected. Five fetuses had no demonstrable VIII:CAg and were regarded as having haemophilia A. The values obtained are presented in Table II.

Of the 11 presumably healthy fetuses 8 were delivered at term and analysis of their blood confirmed the prenatal diagnosis. The mothers of the other 3 are still pregnant. The 5 women whose fetuses were judged as being affected chose to terminate the pregnancy and in all of them the prenatal diagnosis was confirmed by analysis of the blood obtained from the umbilical cord at abortion.

Haemophilia B. The foetal blood sample was uncontaminated in 1 case, but contaminated with amniotic fluid in the other 2, for which reason correction had to be made for the dilution. In one of them IX:Ag could be demonstrated in the amniotic fluid.

All 3 fetuses had IX:Ag <0.05 U/ml and were judged as affected. All three pregnancies were interrupted and in 2 of them the prenatal diagnosis could be confirmed, while no blood was obtained for analysis in the third.

DISCUSSION

Contamination of foetal blood samples with thromboplastic substances in the amniotic fluid can interfere with determination of VIII:C and of IX:C with conventional coagulation assays. Uncontaminated foetal blood is an indispensable condition for the use of such methods. In our material 5 of the 16 samples from fetuses at risk for haemophilia A and 2 of the 3 samples from fetuses at risk for haemophilia B were contaminated with amniotic fluid. But in the remaining cases, it was not possible to exclude a certain dilution with amniotic fluid, which complicates or precludes conventional coagulation analysis of VIII:C and IX:C, respectively.

Dilution of foetal blood samples with amniotic fluid makes it difficult also to assess VIII:CAg and IX:Ag. In addition to determination of EVF in the foetal blood sample and correction of the coagulation analysis to an estimated value of 37% it proved useful, in haemophilia A, as an internal standard, to determine VIIIR:Ag (factor VIII related antigen, von Willebrand factor) and

the ratio VIII:CAg/VIII:R:Ag. The von Willebrand factor in plasma is linked to factor VIII, and in haemophilia A it occurs in a normal amount, but is missing or functionally defective in von Willebrand's disease.

Foetoscopy samples should not be diluted more than 5 times, This range includes a good safety margin for the sensitivity of the method without risk of overdiagnosis. This applies especially to all cases of haemophilia B in which small amounts of IX:Ag can be demonstrated in the amniotic fluid. Such IX:Ag probably originates from the maternal plasma.

Several genetic variants of haemophilia A are known. In severe haemophilia A (VIII:C <1%) it has rarely been possible to demonstrate VIII:CAg, which suggests that the protein is missing or that it is structurally and antigenically severely changed. In moderate (VIII:C 1-4%) and mild (VIII:C 5-25%) forms of the disease 3 subgroups can be recognized: Type I lacks demonstrable VIII:CAg, type IIa has VIII:CAg in proportion to VIII:C and type IIb has VIII:CAg in excess (14).

Some cases classified as moderate have a clinical picture of severe haemophilia A. In such cases prenatal diagnosis may be considered, but at present only members of families with type I of the disease can be offered such an examination. The assumed VIII:CAg levels in an affected foetus with type IIa or b cannot be distinguished with certainty from that in a normal foetus. In the present study prenatal diagnosis could not be offered in 2 cases of moderate haemophilia A because the affected members of the two families were classified as having type IIa and IIb, respectively.

Also haemophilia B has a complex pattern of genetic variants. By the absence, reduced or normal amount of IX:Ag, it is possible to distinguish between CRM-, CRM_R and CRM+ (cross reacting material negative/reduced/positive). Investigations of haemophilia B families have shown that at least 80% of all families with severe and moderate haemophilia B belong to the

CRM- variant in which prenatal diagnosis is possible (15). In our material all the haemophilia B families were CRM-. In both haemophilia A and B, determination of the genetic variant of the disease in living affected members of the family, is absolutely necessary for reliable evaluation of the foetal sample.

Exclusion of the possibility of a woman being a carrier of haemophilia still offers problems. About 75% of all genetically obligate carriers have subnormal levels of factor VIII and IX, respectively, while 25% have normal values. This means that though laboratory studies can detect genetically possible carriers in cases with a subnormal content, they can never exclude the possibility of a woman being a carrier.

About one fourth to one third of all cases of haemophilia are sporadic, i.e. the first case in an apparently unaffected family. To what extent such cases are due to new mutations or to old mutations inherited through the women in several generations is debatable. Carriers who are unaware of their condition can naturally not be offered genetic counsel. This is one of the reasons why prenatal diagnosis can never eradicate the haemophilic disorders.

In our material prenatal diagnosis showed 11 of the 16 male fetuses at risk for haemophilia A to be unaffected and thereby prevented interruption of the pregnancy. But evaluation of 3 fetuses at risk for haemophilia B, showed all to be affected. Of the 19 cases the diagnosis was later confirmed in 15; three women are still pregnant and in one case no blood was obtained for confirmative examination. Prenatal diagnosis can be considered so reliable that it should be included in genetic counselling in severe and moderate haemophilia. The advanced sampling technique and the analysis of the relevant coagulation factors are hitherto available at only a few places in the world. In Scandinavia there are experienced foetoscopic teams in Copenhagen and in Lund, while the laboratory diagnosis has been elaborated and is well established in the Coagulation laboratory in Malmö.

In Sweden there are 167 known cases of severe and 90 cases of moderate haemophilia. The number of carriers is presumably twice as large, i.e. 500-600. Though haemophilia is most often accessible to treatment, the number of requests for prenatal diagnosis shows the need of such an examination. The future frequency of requests for prenatal diagnosis of haemophilia in Sweden will probably be about 10-15 per year.

VIII:CAg	(n = 12)	19 - 60	(M:32) U/dl
VIII:R:Ag	(n = 12)	51 - 183	(M:110) U/dl
Ratio VIII:CAg/ VIII:R:Ag		0.19 - 0.44	(M:0.31)
IX:Ag	(n = 8)	1.8 - 10	(M:4.7) U/dl

Table I. Reference values for VIII:CAg, VIII:R:Ag, ratio VIII:CAg/VIII:R:Ag and IX:Ag in normal foetal samples obtained at 16-20 weeks of gestation. Values corrected to a fetal EVF of 37%.

Prenatal diagnosis	VIII:CAg U/dl	VIII:R:Ag U/dl	Ratio VIII:CAg/ VIII:R:Ag
Normal (n = 11)	(8) * 23-48 (M:32)	(39) * 51-177 (M:108)	0.18-0.45 (M:0.31)
Haemophilia A (n = 5)	< 0.5	24-72 (M:60)	-

* Sample with uncertain degree of dilution - ratio within the normal range.

Table II. Values for VIII:CAg, VIII:R:Ag and ratio VIII:CAg/VIII:R:Ag in 16 fetuses at risk for haemophilia A.

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IMMUNORADIOMETRIC ASSAY OF INHIBITORS OF ANTIHAEMOPHILIC FACTOR A

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ABSTRACT. Ljung, R. and Holmberg, L. (Department of Paediatrics and Coagulation Laboratory, Malmö General Hospital, Malmö, Sweden). Immunoradiometric assay of inhibitors of antihæmophilic factor A. *Acta Paediatr Scand*, 71, 1982.—An immunoradiometric assay (IRMA) for determination of antibodies against f. VIII:C in hæmophilia A was developed. The assay was based on competitive binding of radiolabelled anti-VIII:C and antibodies in the test material to immobilized VIII:C. Fifteen hæmophiliacs with known inhibitors were investigated with the new method and with a conventional neutralization test. In 3 cases the inhibitors were detected only with the IRMA and in the other 12 there was good agreement between the inhibitor levels found with the two methods. It was also possible to demonstrate the antibodies in three non-hæmophilic patients with acquired inhibitors. The IRMA, which can detect the antibodies down to a concentration of 0.02 inhibitor units per ml, is more sensitive than conventional neutralization tests and is thus of practical importance in the investigation of patients with low inhibitor titres.

KEY WORDS: Antihæmophilic factor A, immunoradiometric assay, anticoagulants

Substitution therapy with f. VIII concentrates has dramatically improved the prognosis in hæmophilia A. The development of antibodies against factor VIII is a dreaded complication since it makes specific treatment difficult or even impossible. The reported frequency of this complication varies widely. In Sweden antibodies have been found to appear in about 5% of the hæmophilia A population. Regular examination for such anticoagulants is very important in the care of patients with hæmophilia A. Circulating anticoagulants can be demonstrated by neutralization tests which exist in several modifications (1, 2, 3). Such tests are, however, subject to methodological difficulties and vary in precision and sensitivity. For instance, low antibody titres may escape detection. An immunoradiometric assay for determining of f. VIII antibodies and its clinical application, are described below.

CLINICAL MATERIAL

Citrated plasma was obtained from 15 hæmophiliacs with known inhibitors of antihæmophilic factor A (f. VIII:C), from 10 multitransfused patients with severe hæmophilia A without known anticoagulants and from 10 normals. Plasma was also obtained from 3 non-hæmophilic patients who had spontaneously developed antibodies against f. VIII:C, so-called spontaneous antibodies.

MATERIALS AND METHODS

Quantitative determination of inhibitors by neutralization assay was performed according to Holmberg & Nilsson (2). In this method, various dilutions of the plasma to be tested and a blank were incubated with a f. VIII concentrate for 2 h. After incubation the residual f. VIII activity (f. VIII:C) is determined in a one-stage recalcification system. The inhibitor activity of plasma is expressed as the number of units of f. VIII (1 U of f. VIII is defined as the amount of f. VIII present in 1 ml pooled normal plasma) inactivated by 1 ml of the plasma.

Immunoradiometric assay

Source of antibody. Plasma from a hæmophiliac with a

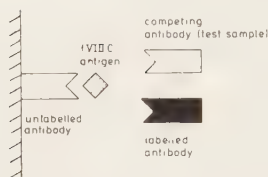


Fig. 1. The IRMA is based on a competition between antibodies for immobilized f. VIII:C antigen.

very high titre of antibodies against f. VIII:C (1000 U/ml) was used as source of antibody.

IgG was isolated and labelled with ^{125}I as described earlier (4, 5). ^{125}I -labelled specific antibodies against f. VIII:C were purified in the way described in detail elsewhere (4). The procedure includes complex formation with a f. VIII concentrate in free solution, isolation of the complex by Sepharose 6 B chromatography and dissociation of the complex by chromatography on Sephadex G-200 at pH 2.4.

The test was based on the principle of a competition, between the labelled antibodies and the antibodies in the test sample, for f. VIII:C antigen (f. VIII:CAG) (Fig. 1), which had been immobilized in the following way: Polystyrene tubes (Ellerman tubes, Medart AB, Sweden) were coated with unlabelled IgG prepared from the immune serum by incubation of 0.6 ml of a dilution 1:500 in 0.1 M carbonate buffer, pH 9.6, for 24 h at $+4^\circ\text{C}$. The tubes were washed three times with 0.01 M phosphate buffer, 0.1 M NaCl, 0.1% bovine serum albumin (BSA), pH 7.4. f. VIII:CAG was then fixed to the tube walls by incubating 0.4 ml of a mixture of plasma from 20 normal persons, diluted 1:8 with 0.01 M phosphate buffer, 0.1 NaCl, 6%

Table 2. Inhibitor levels in 3 patients with spontaneous inhibitors measured by immuno-radiometric assay (IRMA) and neutralisation assay (NA)

Patient no.	IRMA (U/ml)	NA (U/ml)
1	31	200
2	11	30
3	Not parallel	80

BSA, pH 7.4 for 24 h at $+37^\circ\text{C}$. The tubes were then washed again three times as above.

For the competition step equal volumes of the labelled antibody solution (0.2 ml with approximately 9000 cpm) and the plasma or IgG to be tested, in serial dilutions, were mixed. Normal IgG (KABI) 0.5 mg/ml was added to the mixture. After reincubation for 24 h at $+37^\circ\text{C}$ and washing twice with 1% Tween 80 and twice with distilled water the tubes were counted for 1 min in a gamma counter.

IgG was prepared from the plasma samples by Protein-A-Sepharose chromatography (4). The plasmas and IgG fractions were tested in duplicate in seven suitable dilutions.

RESULTS

A reference curve was constructed with the inhibitor plasma from which the labelled anti-VIII:C was purified. Fig. 2 shows the curve obtained when the inhibitor plasma or IgG fraction in various dilutions was allowed to compete with the labelled antibodies for factor VIII:C bound to the tubes. 100% inhibition means that the radioactivity recorded was indistinguishable from that of the blank. The blank value was obtained by addition of radio-labelled antibodies to tubes, which had been incubated with buffer instead of plasma dilution in step 2 and thus contained no antigen to which the specific antibodies could be bound (around 200 cpm or 2% of the added radioactivity). Zero inhibition means that the radioactivity was the same as when, instead of competing antibodies, buffer control had been added in step 3 (around 3000 cpm or 30% of added radioactivity). The reference curve was used to determine the inhibitor levels in other inhibitor plasmas. The reference plasma was set to have 1000 inhibitor U/ml by the IRMA

Table 1. Inhibitor levels in 15 haemophilia A patients measured by immunoradiometric assay (IRMA) and neutralisation assay (NA)

Patient no.	IRMA (U/ml)	NA (U/ml)
1	0.09	0
2	0.09	0
3	0.55	0
4	0.18	0.9
5	0.21	0.7
6	0.46	0.4
7	0.60	4.1
8	0.90	0.4
9	0.90	0.6
10	1.10	3.5
11	1.40	1.1
12	0.50	3.1
13	13	13.0
14	51	70
15	71	72

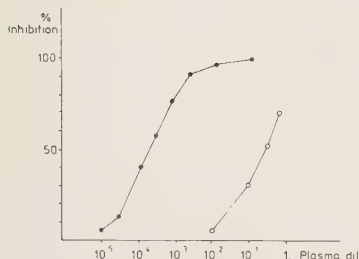


Fig. 2. Inhibitor curves for reference plasma (●—●) and IgG from a haemophilic (patient 3) who had had no detectable inhibitor for 5 years by neutralization assay (○—○).

since this was the titre obtained by our neutralization assay (corresponding to about 2000 Bethesda units).

The reference curve was linear in dilutions 1:800–51 200 (logit log plot; $r=+0.994$), whether it was constructed with the inhibitor plasma or IgG fraction. The lowest inhibitor concentration that could be measured was 0.02 U/ml. To test the accuracy at this level (dil. 1:51 200), the reference plasma was tested 15 times and the values obtained (mean $\pm$ 2 SD, 2508 ± 182 cpm) were statistically different (t -test, $p < 0.001$) from the blank values (2766 ± 158 cpm). The intra-assay coefficients of variation measured in dilutions 1:800 and 1:51 200 were $< 5\%$.

Low dilutions (1:1–1:4) of plasma and IgG-depleted plasma (by Protein-A-Sepharose) from normals and haemophiliacs without known inhibitors gave a slight inhibition of radiolabel binding, apparently not related to the presence of specific antibodies.

This inhibition was not found with the IgG preparations from the same plasma samples. Plasma was therefore tested in 1:8 and higher dilutions. When patients with low inhibitor titre or no inhibitor in neutralization assay were tested, IgG from their plasma was assayed instead of whole plasma.

Table 1 gives the inhibitor levels measured both by IRMA and the neutralization assay in 15 patients with severe haemophilia A. All the

patients are known to have or to have had an anticoagulant. Patient 1 had had no detectable inhibitor by neutralization assay for nearly 1 year, patient 2 for a couple of months and patient 3 for 5 years (Fig. 2). The IRMA, however, demonstrated an inhibitor in all these cases. All the dilution-curves obtained for the patients' IgG were parallel to the reference curve.

Table 2 shows the inhibitor levels measured by IRMA and the neutralization assay in three patients with spontaneous inhibitors. The dilution curve in patient 3 was not parallel to the reference curve (Fig. 3).

When IgG from 10 normals and 10 multi-transfused haemophiliacs without known inhibitor was tested, neither method showed any inhibitor activity.

DISCUSSION

The detection of anticoagulants against f. VIII:C has hitherto always relied on neutralization assays (1, 2, 3). The present method is an immunoradiometric assay based on a haemophilic antibody and has a sensitivity down to an inhibitor titre of 0.02 U/ml.

When 15 haemophilic patients with known inhibitor were tested, good agreement was found between the results obtained by IRMA and neutralization test. In patients 1 and 2 it had not been possible, on repeated neutraliza-

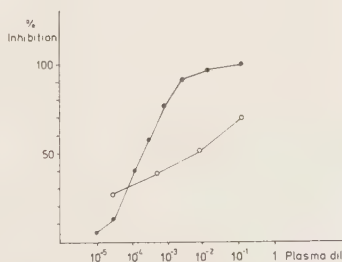


Fig. 3. Inhibitor curves for reference plasma (●—●) and plasma from a non-haemophilic with a spontaneous inhibitor (○—○).

tion tests for nearly one year in patient 1 and for a couple of months in patient 2, to detect antibodies. The patients were examined on two occasions with IRMA and their inhibitors were detected on both. Since the IRMA is more sensitive it detects a developing anticoagulant early in a patient.

In patient 3 it had not been possible for 5 years to demonstrate an inhibitor with neutralization assay, but IRMA revealed antibody titre of 0.55 U/ml. The large discrepancy between the results of the methods might be due to the properties of the patient's antibody. It might be directed against antigenic sites located remote from the biologically active site of the molecule and thus not be detectable with a neutralization test but only with an assay based on competitive binding of antibodies. The existence of different anti-f. VIII:C antibodies, one type being directed against the active site and a second against other parts of the molecule, has been suggested earlier (6). An IRMA might be a valuable tool for discovering antibodies with low affinity for the biologically active site of the f. VIII molecule. Such antibodies may herald the appearance of frankly neutralizing antibodies.

Good agreement was found between IRMA and neutralization assay values in 7 haemophilia A patients with rather high antibody titres (>4 Bethesda units/ml) according to a recent, similar method (7). As pointed out in that study, the presence of f. VIII:CAG in a sample interferes with the assay and gives false inhibitor values. This was evident in our study when we tested normal plasma instead of haemophilic plasma.

But we have also noticed a slight and apparently unspecific inhibitory effect in low dilutions of plasmas and IgG-free plasmas from haemophiliacs without known inhibitors. In the latter cases it could not be ascribed to the presence of f. VIII:CAG, but seemed to be an unspecific action of plasma proteins. To avoid these errors, we chose to test the IgG fraction in cases where low inhibitor values could be expected. The IgG prepared by Protein-A-Sepharose is devoid of the IgG:3 subclass and such antibodies are therefore not determined. Typing of f. VIII antibodies has, however, shown a clear predominance of the IgG:4 subclass (8, 9, 10).

When IgG from 3 non-haemophilic patients who had spontaneously developed an inhibitor of factor VIII:C, i.e. spontaneous or acquired inhibitor, were examined, a dose-response curve parallel with the reference curve was found in two of the patients and a non-parallel curve in the third. The lack of parallelism probably reflects different affinity properties between the competing antibodies. The spontaneous antibody from patient 1 and the reference antibody have both been utilized in IRMA:s for f. VIII:CAG, and good agreement of the f. VIII:CAG levels was found between the two methods (4, 5).

The results obtained with the IRMA are to a certain extent dependent on the properties of the labelled antibody. Other antibodies used for the labelling and construction of a reference curve might give somewhat different results. The IRMA described here for determining of anticoagulants in haemophilia A is more sensitive than neutralization tests and thus of practical value in the investigation of cases with low inhibitor titres, and perhaps in cases with antibodies of unusual specificity and for the early detection of an anticoagulant. When such antibodies are detected prophylactic treatment should be discontinued.

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